REGULATION OF *French and* THIOPHENE METABOLISM *African Marigolds* IN TAGETES SPECIES

JOHN J.M.R. JACOBS

REGULATION OF THIOPHENE METABOLISM IN *TAGETES* SPECIES

FRENCH AND AFRICAN MARIGOLDS

REGULATION OF THIOPHENE METABOLISM IN *TAGETES* SPECIES

French and African marigolds

een wetenschappelijke proeve op het gebied
van de natuurwetenschappen, in het bijzonder de biologie

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aan mijn ouders,
voor mijn jeugd op
"Gen Dreesje"

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VOORWOORD

Promoveren is leren. En ik heb veel geleerd, de afgelopen vijf jaar. Niet alleen over plantjes en stofjes en DNA, maar ook over mensen en afdelingen en projecten en (Engels) schrijven. Een project is een project, en een proefschrift is een proefschrift, en daartussenin zitten een zee van tijd (die voorbij vliegt), grootse plannen, nieuwe plannen, besprekingen, experimenten, nieuwe besprekingen en nog meer experimenten. Uiteindelijk komt er iets uit, anders dan eerst gedacht, maar toch wel leuk. Dit is het leuke van onderzoeken: dat het creatief is en onvoorspelbaar. Ik wil graag iedereen bedanken die mij de afgelopen jaren iets heeft bijgebracht, misschien zonder het te weten. Te beginnen met George, de promotor, dan de medewerkers van de afrikaantjes-groep: Ton, Randy, Margaret, Anke, Joan, Clementine; vervolgens de studenten: Peter, Andreas, Ignas, Leonard, Edward, Jorn, Willem, Leonie, Zeban, Marc, Karin. De medewerkers van de kassen, de bibliotheek, grafische vormgeving, en fotografie dank ik voor hun goede diensten en prompte service bij mijn vaak chaotische en raadselachtige verzoeken. Tenslotten bedank en groet ik alle medewerkers van de vakgroep (boven en beneden, oude en nieuwe), voor de gezelligheid en de hulp bij vele kleinigheden (faxen, mailen, blotten, printen, prakken, pruttelen, etc., etc.). Wij zien elkaar nog!

ABBREVIATIONS

Thiophenes and polyacetylenes

$AcOCH_2BBT$	5-(but-3-en-1-ynyl)-2,2′-bithiophenyl-5′-methyl acetate
BBT	5-(but-3-en-1-ynyl)-2,2′-bithienyl
BBTOAc	5-(4-acetoxy-1-butynyl)-2,2′-bithienyl
$BBT(OAc)_2$	5-(3,4diacetoxy-1-butynyl)-2,2′-bithienyl
BBTOH	5-(4-hydroxy-1-butynyl)-2,2′-bithienyl
$BBT(OH)_2$	5-(3,4-dihydroxy-1-butynyl)-2,2′-bithienyl
BBTOHOAc	5-(4hydroxy-3-acetoxy-1-butynyl)-2,2′-bithienyl
BPT	2-(but-3-en-1-ynyl)-5-(penta-1,3-diynyl)-thiophene
$HOCH_2BBT$	5-(but-3-en-1-ynyl)-[2,2′]-bithiophenyl-5′-methanol
HPT	2-(hex-5-en-1,3-diynyl)-5-(prop-1-ynyl)-thiophene
MeBBT	5-(but-3-en-1-ynyl)-2,2′-bithiophenyl-5′-methane
PYE	trideca-3,5,7,9,11-pentayn-1-ene
α-T	2,2′:5′,2"-terthienyl

Others

BAP	6-benzylaminopurine
EMS	ethyl methane sulfonate
HPLC	high performance liquid chromatography
NAA	1-naphtalene acetic acid
PCR	polymerase chain reaction
PR	pathogenesis related
TPC	*Tagetes patula* cDNA
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER 1

FUNCTION AND REGULATION OF SECONDARY METABOLISM

INTRODUCTION

Metabolism is the complex network of enzyme-catalyzed conversions of cellular constituents that occur in a regulated and coordinated fashion in living organims. Already in 1891 (before the concept of metabolism was truly understood), the plant physiologist Kossel proposed to set apart compounds that occur incidentally in cells or are not absolutely required for life, from cellular constituents which occur without exception in viable cells. The latter he called primary, the former secondary compounds (Kossel, 1891). Since then, a consistent definition covering the immense range of secondary compounds has been elusive. In one, rather pragmatic, definition, a secondary metabolite is a substance that appears to have no explicit role in the internal economy of the organism that produces it. In another approach, primary and secondary metabolism are separated on basis of their distribution. Primary metabolism, then, comprises the compounds and enzymatic conversions that occur with little variation in the majority of cells of most living organisms, whereas secondary metabolism comprises the enzyme-catalyzed reactions and their products which are confined to a limited number of species and only occur in a restricted number of cells during a limited phase of an organisms life time. Although secondary compounds may not be of immediate importance for a cell's survival, they have been shown to perform important functions at the cellular, organismal and ecological level (Harborne, 1988; Koes *et al.*, 1994; Rhodes, 1994). Furthermore, some secondary compounds occur almost ubiquitously, at least among plants. Therefore, the separation between primary and secondary compounds is diffuse. Secondary metabolism is active in microorganisms (which produce a great variety of antibiotics) and animals, but plants are by far the most productive and versatile producers of natural products. It has been postulated that plants were forced during evolution to develop extensive metabolic capabilities to compensate for their sessility (Bell, 1981; Wink, 1988) and lack of an immune system (Williams *et al.*, 1989). This introduction will be concerned exclusively with secondary metabolism of plants.

Secondary plant compounds greatly influence our perception of the natural environment. An important structural component of wood, lignin, is a secondary compound. The *Blue* Mountains in Australia and the *Smoky* Mountains of the USA probably derived their names from the atmospheric scattering of blue light by plant-derived volatile terpenes. The release of volatile secondary compounds by plants has been estimated to contribute significantly to smog and other forms of air pollution (Went, 1974). However, usually secondary compounds occur in trace amounts. As such they are responsible for the taste of natural foodstuffs and for the colour and fragrance of flowers and fruits. Many are used in medicine, *e.g.* alkaloids, cardiac glycosides, antibiotics, and some have addictive properties, *e.g.* nicotine, caffeine, cannabinol, cocaine, morphine. Others are used as flavouring additives to foodstuffs, as odoriferous components of perfumes, or as dyes in cosmetics and textile colouring. Yet others which can be gained in large amounts are utilized as raw materials in industry, *e.g.* rubber, tannins, and cellulose.

Despite the omnipresence of natural products, basic biochemical and molecular genetic understanding of secondary metabolism has progressed relatively slowly during the last few decades. This was largely due to the restricted distribution and low abundance of most secondary compounds combined with the bewildering diversity and complexity of structures observed. Whereas only several hundreds of primary metabolites are turned over in primary metabolic pathways, over 100,000 secondary compounds have been identified over the last century and certainly many more will be identified in the future. Progress in our knowledge of secondary metabolism was furthermore slow due to a lack of interest. These compounds seemed to serve no important functions and occurred (due to breeding) at negligible levels in economically important crops. However, already in the last century it was postulated that secondary plant products might be important in the deterrence of herbivores (Stahl, 1888). From the late fifties onwards, the insight has grown that secondary compounds do have important ecological roles, and nowadays it is generally accepted that, though the biosynthesis of secondary compounds may

be without significance to the individual producer cell, it does play a decisive role in the development and survival of the producer organism as a whole.

GENERAL CHARACTERISTICS

Secondary metabolic pathways start from precursors of primary metabolism and are essentially extensions or branches of primary pathways. This is illustrated well by the shikimate pathway, which extends into various pathways of secondary metabolism (Poulsen and Verpoorte, 1991). A competition exists between primary and secondary metabolic routes for substrates, energy, and reducing power. At the cellular level, competing pathways may be separated in different compartments which allows them to operate and be regulated relatively independently. Also enzymes and intermediates of a single pathway may be targeted to different cellular compartments, thereby providing a means of regulation and contributing to spatial directionality.

Conversions of secondary metabolism are carried out by specific enzymes encoded by specific genes. The enzymes of secondary metabolism are functionally similar to those of primary metabolism and comparative evidence in some cases suggests that they have evolved from enzymes of primary metabolism (Koes *et al.*, 1994). In many secondary pathways, a relatively limited number of core compounds are formed which subsequently give rise to a huge array of derivatives through various modifications (hydroxylation, (de-)methylation, acylation, glycosylation). It has been proposed that the enzymes of the "core pathways" are more selective with respect to their substrates than the modifying enzymes, which may use a range of substrates, though usually retaining stereo selectivity. Whereas the activities of the "core enzymes" occur in an ordered linear sequence, those of the modifying enzymes often can be depicted in "metabolic grids", *i.e.* polydimensional networks of reactions leading with different rates to the same products (Luckner, 1990). The purpose of various modifications may be to extend the range of active compounds,

inactivation, or targeting to storage compartments.

The accumulation patterns observed of any secondary compound in various parts of a plant are the result of synthesis, transport, storage, conversion, degradation, and chemical stability. The importance of transport at the organismal level may be illustrated by the examples of nicotine and the tropane alkaloids which are both synthesized exclusively or predominantly in the roots and exported into the aerial parts of the producing plants (Luckner, 1990; Hashimoto *et al.*, 1991).

Secondary compounds may occur freely in the cytoplasm or may be stored in specific compartments. Vacuoles are the most important intracellular storage spaces for soluble compounds. Often, compounds are made more soluble (*e.g.* by glycosylation) to make storage in vacuoles possible. The other major intracellular storage entities are plastids which generally contain relatively insoluble compounds (*e.g.* carotenoids in chromoplasts of ripening fruits). Many relatively insoluble secondary compounds are secreted into the extracellular space where they accumulate in the cell wall (phenolics, lignin, tannins), or in the subcuticular space and cuticle (terpenoids, waxes).

Although secondary compounds usually accumulate in specific tissues, the cells of these tissues normally do not show obvious morphological adaptations. Two types of specialized cells for synthesis and/or storage of secondary compounds that are easily distinguishable by their morphology are laticifers and glandular cells. Laticifer cells of leaves are surrounded by parenchymatic tissue and contain many vacuoles in which large amounts of soluble secondary compounds may be stored. In *Catharanthus roseus*, it has been shown that alkaloids are synthesized in the surrounding cells and subsequently transferred to the laticifers for storage (Müller, 1976; Müller *et al.*, 1976). Glandular cells are often epidermal structures occurring in the form of hairs (trichomes) that excrete into the subcuticular space and cuticle. Internal glands that excrete into cavities are also known. The morphology of glands is as variable as the products they excrete, which may be (mixtures of) terpenoids (essential oils, resins), flavonoids, and fatty oils (waxes).

FUNCTIONS OF SECONDARY COMPOUNDS

Secondary compounds have long been considered merely by-products of an unbalanced metabolism or detoxification products of harmful compounds. However, in view of the present knowledge of the biochemical specificity and sophistication, and the precise temporal and spatial regulation of many secondary pathways this hypothesis is no longer tenable. Over the years an immense body of information has accumulated demonstrating numerous ecological functions of secondary compounds (Harborne, 1988). Secondary compounds are implicated in environmental stress protection and in various defence strategies against pathogens (fungi, bacteria, viruses), predators (insects, mammals), and competing plants. They may act as attractants of pollinators and seed dispersers (colour, fragrance, and taste of flowers and fruits). At the cellular level they are involved in signal transduction within plants and between plants and parasitic or symbiotic microorganisms. Biochemical and genetic evidence pertaining to these functions is accumulating rapidly and genetic manipulation offers new means of convincingly proving various hypotheses. Some examples will be discussed here.

In the perpetual combat against pathogenic bacteria and fungi, plants exploit various defence systems in which secondary compounds play a major role. Some defensive barriers are continuously erected (*e.g.* anti-feedants), whereas others are raised in response to pathogen attack. In the latter type of defence, secondary compounds are involved both as a weapon and as a signalling compound.

Phytoalexins are low molecular weight, anti-microbial compounds that are synthesized by a plant after exposure to microbes (Dixon, 1986; Ebel, 1986). Phytoalexin synthesis is elicited by specific pathogen-derived compounds termed elicitors. A range of secondary compounds qualify as phytoalexins, *e.g.* sesquiterpenes, furanocoumarins, stilbenes, isoflavonoids, polyacetylenes, and glucosinolates (Rhodes, 1994). Direct evidence of the function of these compounds has been obtained in few cases only. Stilbene synthase (STS)

catalyses the condensation of *p*-coumaroyl CoA and malonyl CoA to the stilbene resveratrol. Genetic transformation of tobacco, which does not contain this gene, with the STS gene from *Vitis vinifera* (grape vine) resulted in the rapid appearance of high levels of STS mRNA and the accumulation of large amounts of resveratrol upon fungul infection. This in turn correlated with a reduced incidence of successful infection by *Botrytis cinerea* (Hain *et al.*, 1993).

Various secondary compounds have been implicated in signal transduction during pathogen infection (Enyedi *et al.*, 1992). Salicylic acid, a phenylpropanoid derivative, was shown to act as a messenger in the establishment of systemic acquired resistance (SAR) upon viral infections of tobacco and cucumber (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Salicylic acid is synthesized upon infection and rapidly transported. It induces the expression of defence-related genes (Ward *et al.*, 1991), and a mechanism for *in planta* inactivation of salicylic acid has been proposed (Rhodes, 1994). A mechanism for inactivation may be important to prevent persistence of the signal after it has effected its response. Transgenic plants expressing a bacterial gene that codes for an enzyme which degrades salicylic acid do not exhibit SAR (Gaffney *et al.*, 1993). Jasmonic acid is another, lipid-derived, secondary compound that meets the requirements of a signal molecule. It is induced upon wounding and treatment with fungal extracts, it is transported and specifically induces the expression of proteinase inhibitors and genes of secondary pathways (Creelman *et al.*, 1992; Gundlach *et al.*, 1992; Mueller *et al.*, 1993). The related methyl-jasmonate, being volatile, has even been implicated in airborne inter-plant communication (Farmer and Ryan, 1990).

A ubiquitous environmental stress factor to which plants are exposed, is UV light. Flavonoids efficiently absorb UV light and are therefore good candidate UV protectants. Upon UV irradiation, transient transcriptional activation of flavonoid biosynthetic genes occurs, mainly in epidermal cells, leading to accumulation of flavonoids in the epidermis (Schmelzer *et al.*, 1988; Kubasek *et al.*, 1992). This suggests that flavonoids do indeed function as a protective shield. The growth of *Arabidopsis* mutants which are unable to synthesize

flavonols was strongly retarded when placed under short wavelength UV light, thereby directly demonstrating the role of flavonoids in UV protection (Li *et al.*, 1993).

Most flower pigments are flavonoids or carotenoids. In insect pollinated plants, these pigments act as a visual signal to attract pollinating insects and birds (Waser and Price, 1983), although probably also oderous volatiles are involved in this attraction. Flowers from which the petals have been removed, and flowers of a homeotic mutant in which the coloured petals are transformed into green sepal-like structures are much less visited by insects than normal flowers (Ford and Gottlieb, 1992). The regulation of pigment accumulation in flowers has been studied profoundly in the case of anthocyanins. These pigments accumulate predominantly in the upper epidermis just prior to flower opening, which is compatible with a role as a visual signal. Accumulation is primarily controlled by modulation of the transcriptional activity of the structural genes of anthocyanin synthesis (Martin and Gerats, 1993).

Flavonoids play an important role in anther and pollen development and fertilization. Flavonoid biosynthetic genes and enzymes are active in developing anthers and pistils, leading to accumulation of anthocyanins, flavonols, and chalcones. Transgenic petunia plants in which expression of the chalcone synthase gene is reduced produce unpigmented pollen, are sterile in self pollination, and the pollen do not form a functional pollen tube *in vitro* (Van der Meer *et al.*, 1992; Taylor and Jorgensen, 1992). Normal pollen germination and tube growth is restored when the pollen are placed on wild type stigmas or when extracts from wild type stigmas are applied *in vitro* (Mo *et al.*, 1992). The active compounds in the stigmas have been identified as flavonols. The mechanism through which flavonols promote pollen development is unknown, but the low concentrations required suggest a function as signalling compounds.

Another area where secondary compounds may act as signal compounds is the establishment of intimate relationships between plants and bacteria (Long and Staskewicz, 1993). The symbiosis between leguminous plants and

Rhizobium bacteria involves a complex sequence of developmental changes both in the host and the bacterium. Flavonoids released from the host plant have the capacity to activate bacterial Nod genes (Peters *et al.*, 1986; Redmond *et al.*, 1986), probably by binding of the flavonoid signal to a bacterial receptor protein. The spectrum of flavonoids excreted provides a means of determining host specificity (Spaink *et al.*, 1987; Horvath *et al.*, 1987). In the infection of plants by the parasitic *Agrobacterium rhizogenes* and *A. tumefaciens*, acetosyringone and other phenolic compounds (benzoic acid, cinnamic acid) have been characterized as inducers of the *vir* genes (Stachel *et al.*, 1985; Spencer and Towers, 1988), which is required for T-DNA tranfer to the host plant. This last example demonstrates that secondary compounds may be used "against" the producing plant as well.

REGULATION OF SECONDARY METABOLISM

Regulation of secondary metabolism is strongly related to its role *in planta*. Factors determining which regulatory mechanism is most likely to operate in a specific situation are the extent to which a secondary pathway draws precursors from primary pathways, the prevailing or desired concentration of a secondary compound, and the rate at which the concentration of a compound needs to change. The major difference between regulation of primary and secondary pathways pertains to the extent to which it occurs. Though the activity of primary metabolism may vary, it never stops (although exceptions exist, *e.g.* seeds, spores, and dessication tolerant plants which may be completely metabolically inactive). As has been amply illustrated in the previous section, the proper functioning of secondary compounds usually requires precisely controlled spatial and temporal accumulation patterns. The timing and site of secondary compound accumulation are either internally programmed (as in pigmentation and pollen development), or the capacity of accumulation is acquired during development and the actual accumulation is elicited by specific

external signals (as in phytoalexin synthesis). These characteristics qualify secondary compound accumulation as a developmental process.

Development has been defined as the sum of all events that contribute to the progressive elaboration of the body of an organism (Steeves and Sussex, 1989). The essential aspects of development are differentiation and growth. Differentiation encompasses the changes that occur in cells or groups of cells to bring about their irreversible structural and functional distinctiveness from surrounding cells, whereas growth is the irreversible increase in size accomplished by a combination of cell division and cell enlargement. To achieve development, growth and differentiation occur in a highly regulated and coordinated fashion, according to the information that is laid down in the endogenous, inherited genetic program, and to some extent in response to external signals. Developmental processes are ultimatly controlled at the level of gene expression, as has been shown in the case of flower development (Coen and Meyerowitz, 1991; Mandel *et al.*, 1993).

Secondary metabolism has several levels of control. The kinetic (K_m, V_{max}) and allosteric (substrate activation, feed back inhibition) properties of enzymes determine their activity in relation to the concentration of substrates and products. Cellular compartmentation of substrates and enzymes, the rate and capacity of transport and storage, the extent of degradation, and the chemical stability of intermediates and end products contribute to control at a "logistics" level. Mechanisms for covalent modification or degradation of enzymes provide biochemical means of control.

As other aspects of differentiation and development, secondary metabolism is ultimately regulated at the gene level. Moreover, in the best studied secondary pathways this clearly constitutes the primary level of regulation. Genetic regulation is achieved primarily by changing the rate of gene transcription. However, mRNA stability, as well as mRNA translation and post-translational modification also offer opportunities for control. Although, in principle, a metabolic pathway can be controlled by regulating only one gene, usually concerted regulation of several genes of a pathway is observed

(Kubasek *et al.*, 1992). Clearly, this coordinated regulation increases effectiveness of the control mechanism and economy of the pathway. Not surprisingly, secondary compounds have been shown to directly influence the expression of genes involved in their biosynthesis (Loake *et al.*, 1991).

A sophisticated mode of control of secondary metabolism is the utilization of isoenzymes, which is mirrored at the gene level by the existence of gene families. The enzymes encoded by different members of a gene family may be functionally indistinguishable, as in the case of chalcone synthase, or may be true iso-enzymes, as in the case of chorismate mutase. The latter enzyme is, strictly, an enzyme of primary metabolism operating in the shikimate pathway towards aromatic amino acids. Two isoforms of chorismate mutase have been identified (CM1 and CM2) which both catalyze the same conversion. However, CM1 and CM2 have different kinetic and allosteric properties, are targetted to different cellular compartments and are differentially regulated at the genetic level (Poulsen and Verpoorte, 1991). It is hypothesized that two complete shikimate pathways operate in parallel in plants, one supplying the aromatic amino acids and one, activated if needed, supplying precursors for secondary metabolism. In case of chalcone synthase, not the iso-enzymes but the gene promoters represent the major control element. By utilizing differentially regulated promoters, various members of the chalcone synthase gene family are expressed under different conditions and in different tissues, thereby greatly contributing to flexible utilization of chalcone synthase (Koes *et al.*, 1989; Harker *et al.*, 1990).

THIOPHENES

Introduction

Nematodes are highly successful plant parasites that attack many cultivated species, thereby causing annual agricultural losses estimated at 77 billion dollars worldwide (Sasser and Freckman, 1987). Cultivation of *Tagetes* species (marigolds; *Asteraceae*) effectively reduces populations of endoparasitic nematodes (Slootweg, 1956; Oostenbrink *et al.*, 1957). The nematode-suppressing activity of marigolds has been attributed to thiophenes which are present in the roots of marigolds. Thiophenes display nematicidal activity *in vitro* (Uhlenbroek and Bijloo, 1958; 1959), and a range of related thiophene-containing Asteraceous species also suppress nematode populations. The biocidal activity of thiophenes is greatly increased in the presence of near-UV light (Gommers and Bakker, 1988). A proposed mechanism for toxicity involves photoactivation of the thiophenes followed by generation of free oxygen radicals which cause extensive cellular damage. In agreement with this rather unspecific mechanism for toxicity, it has been shown that, *in vitro*, thiophenes are toxic to a range of organisms besides nematodes (Gommers and Bakker, 1988). It is unknown how thiophenes, in the absence of UV light, exert their toxicity in the soil. Since populations of free-living ectoparasitic nematodes are not affected, it seems essential for toxicity that the organisms penetrate the roots.

Thiophenes are fascinating secondary compounds, not only for their presumed ecological role as nematicides, but also because of their chemistry and biosynthesis. Thiophenes are characterized by up to three five-membered sulfur-containing aromatic rings that are linked together by the α-carbons. The carbon skeleton of thiophenes is derived from straight-chain polyacetylenes containing up to five highly unstable conjugated triple bonds, some of which may be preserved in the thiophene molecule. The conjugated triple bonds and the aromatic sulfur atoms identify thiophenes as chemically unique secondary

compounds. Biosynthesis of thiophenes requires the convergence of the pathways of fatty acid/polyacetylene metabolism and of sulfur metabolism.

Sulfur is one of the nine essential macronutritional elements of plants. Within plants, 90% of sulfur is in the cysteine and methionine residues of proteins (Salisbury and Ross, 1992), but it is also essential in sulfolipids of thylakoid membranes, and in various coenzymes (thiamine pyrophosphate, biotin, S-adenosylmethionine, coenzyme A). Sulfur is absorbed as SO_4^{2-} by the roots and transported through the phloem to aerial parts. The sulfate is reduced to sulfide, which is (bound or free) rapidly incorporated into organic sulfur compounds. Adenosine-5'-phosphosulfate (APS) and glutathione are important intermediates in sulfur metabolism. The complete reduction of sulfate can take place in the chloroplasts of green tissues and probably in plastids in the roots. Sulfur metabolism has been reviewed recently (Rennenberg *et al.*, 1990; Schmidt and Jäger, 1992). Besides thiophenes, few other secondary compounds contain sulfur, *e.g.* glucosinolates of crucifers and other species, odoriferous sulfur compounds of onion, garlic, and cabbage, and sulfate esters of flavonoids in *Flaveria* species.

Biosynthesis of thiophenes

Data concerning the chemical structure and distribution of hundreds of polyacetylenes and thiophenes have been compiled in two extensive reviews (Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1985). On basis of these data and scarce feeding experiments, biosynthetic pathways were proposed by Bohlmann and coworkers. These pathways, though broadly acceptable, are not indisputable in details. Especially, the order in which various conversions take place is in most cases not unequivocally determined.

Feeding experiments and chemical similarity indicate that polyacetylene synthesis starts from oleic acid (Bu'Lock and Smith, 1967; Bohlmann *et al.*, 1969) (Fig. 1.1). Progressive introduction of double and triple bonds leads to a

C_{18} methyl-triyne-ene-acid which is shortened by ß-oxidation and reduced to a C_{14} methyl-triyne-ene-ol. This compound still contains the characteristic double bond of oleic acid. Oxidation and water elimination yields a widespread C_{14} methyl-triyne-diene-ol which is subsequently desaturated and shortened by one carbon to yield the widely distributed trideca-(3,5,7,9,11)-pentayne-(1)-ene (PYE) (Bohlmann *et al.*, 1968). By variation in the extent and pattern of desaturation and the mode of chain shortening, a range of other polyacetylenes are formed.

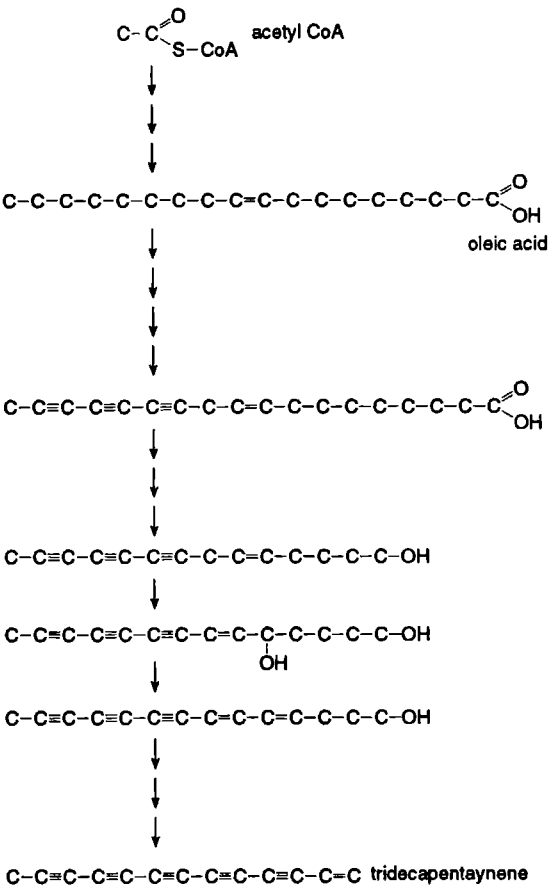

Figure 1.1 Proposed pathway for polyacetylene biosynthesis.

The most intriguing event of polyacetylene biosynthesis is the formation of the triple bonds. In analogy with fatty acid desaturases that introduce double bonds, it is to be expected that distinctive, membrane-bound enzymes are utilized for the introduction of each triple bond.

PYE is the precursor to a large variety of widely distributed C_{13} and C_{12} thiophenes, including the thiophenes of *Tagetes* (Bohlmann and Hinz, 1965; Bohlmann *et al.*, 1966). Synthesis of thiophenes from PYE requires the formation of one to three thiophene rings. In the formation of terthienyls, dehydrogenation of the allyl group is required prior to formation of the third ring. To obtain C_{12} thiophenes, the terminal methyl group must be removed. The biosynthetic connections between the most widely distributed "core" thiophenes related to PYE can be depicted in a three-dimensional metabolic grid in which the directions indicate the "core" reactions: ring formation, desaturation, and demethylation (Fig. 1.2). The most probable sequence of conversions in *Tagetes* and related species (according to Bohlmann and coworkers) is indicated by bold-printed arrows. However, different routes may be favored in various species, and indeed, it cannot be excluded that the order in which the conversions occur is to some degree free within each species.

A range of relatively polar derivatives of PYE and of the "core" C_{12} and C_{13} thiophenes can be obtained by oxidation of either the allyl group (via an intermediate epoxide), or the terminal methyl group (Fig. 1.2). Hydroxy and acetoxy derivatives of the allyl group have been identified in all polyacetylenes and thiophenes that contain this group. Oxidation products (hydroxy, acetoxy) of the methyl group have been identified for MeBBT and Me-αT. Some oxidation products are probably metabolic end products of thiophene synthesis. Others may still be subject to ring formation, dehydrogenation, and demethylation, in which case the metabolic grid (Fig. 1.2) should be extended with several dimensions for proper representation of all possible conversions.

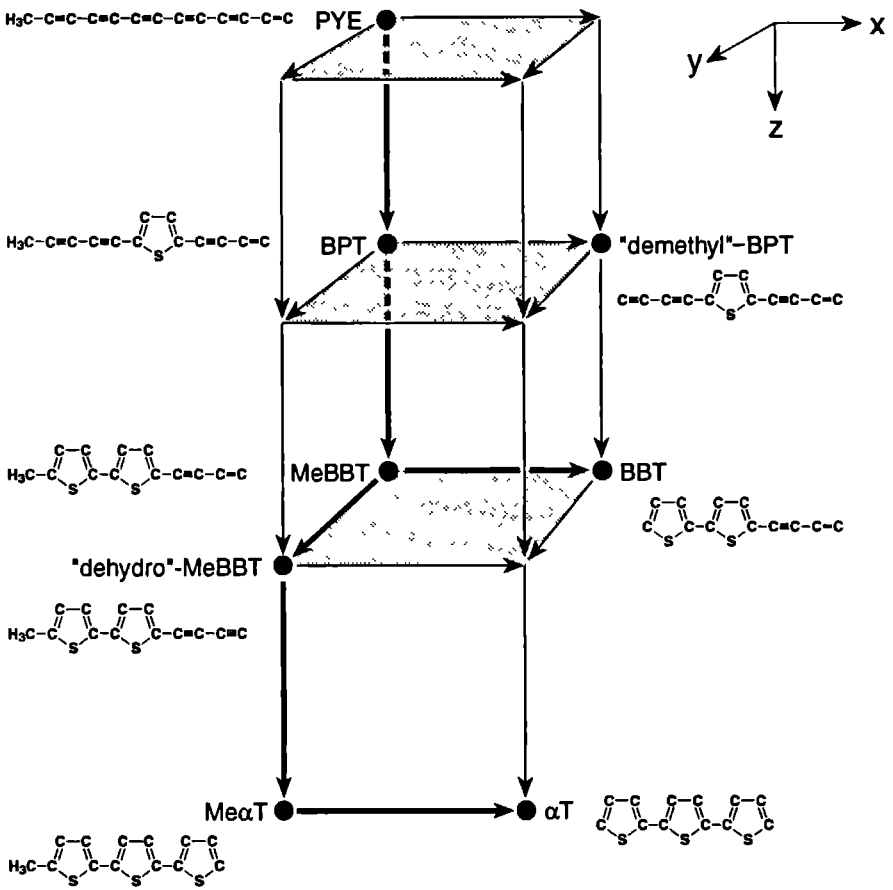

Figure 1.2 Possible biosynthetic routes from PYE to various thiophenes. The directions x, y, and z indicate demethylation, desaturation, and ring formation, respectively. The bold-printed arrows indicate the most probable pathway in *Tagetes*, according to Bohlmann and coworkers.

The key feature in the conversion of PYE to thiophenes is the formation of sulfur-containing aromatic rings. The mechanism of thiophene ring formation is unknown. Based on feeding experiments with PYE and co-occurrence of methyl-thio-ethers and thiophenes, it is assumed that a two-step reaction is involved (Bohlmann *et al.*, 1964; Bohlmann and Hinz, 1965) (Fig. 1.3). First,

H_2S (bound or free) is added to a diyne group, yielding an intermediate thiol-ene group. Subsequently, both methyl-thio-ethers and thiophenes are formed from this intermediate. In principle, four different monothiophenes can be formed from PYE. However, only two monothiophenes occur in plants of which BPT is by far the most widely distributed.

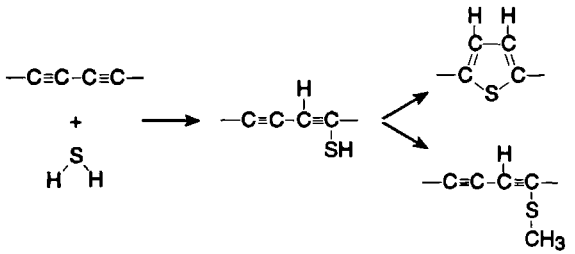

Figure 1.3 Proposed mechanism for thiophene ring formation.

Nothing is known about the enzyme(s) of thiophene ring formation. Are two separate enzymes involved in the formation of a ring (one for H_2S addition and one for ring closure)? Are different enzymes utilized for ring formation in different substrates or positions? The predominance of one monothiophene indicates that the enzyme which forms the first ring has a strong preference with respect to the diyne group to which H_2S is added. On basis of the predominating mono- and bithiophenes, it is assumed that normally the three rings of αT are formed sequentially in the order: central ring, left ring, right ring. However, feeding experiments with *Tagetes patula* showed that αT may be formed from different bithienyl precursors (Jente *et al.*, 1981). This implies that the order of ring formation may vary, and that either the third ring-forming enzyme is not very specific with respect to its substrate, or that distinctive (iso-)enzymes are present. Indeed, it is not sure whether enzymes are involved in ring formation, since the conversion can be accomplished chemically under fairly mild conditions (Schulte *et al.*, 1962).

It is unknown in which cellular compartments biosynthesis of polyacetylenes and thiophenes is localized. In plants, fatty acid synthesis up to oleic acid is localized predominantly or exclusively in chloroplasts and plastids (Harwood, 1988, Sparace *et al.*, 1988). Fatty acid desaturation occurs both in chloroplasts/plastids and on the membranes of microsomes and the endoplasmatic reticulum. Peroxisomes, and possibly mitochondria, are the site of fatty acid degradation by ß-oxidation (Harwood, 1988; Cheesbrough, 1989). Feeding experiments indicated that triple bond formation takes place in a particulate, plastid-containing, fraction (Bohlmann and Schultz, 1968). All taken together, several cellular compartments are probably involved in the total biosynthesis of thiophenes. However, of all compartments involved, plastids are probably the most important.

Marigolds

Probably due to the long-recognized nematode-suppressing ability of these ubiquitous garden flowers, French and African marigolds (*Tagetes patula* and *T. erecta*, respectively) have become the favorite model plants for investigations on thiophene synthesis. Indeed, the first naturally occurring thiophene identified was isolated from *T. erecta* (Zechmeister and Sease, 1947). The major thiophenes of *T. erecta* and *T. patula* (Fig. 1.4) are derivatives of BPT. Thiophenes not related to BPT have been reported occasionally. No PYE, nor any other polyacetylene, nor BPT have been isolated from *T. patula* or *T. erecta*.

Relatively few investigations have been concerned with the regulation of thiophene synthesis in intact plants. Thiophenes accumulate during plant development predominantly in the roots of *Tagetes* species, but have also been isolated from aerial parts, especially stems and flower heads (Downum and Towers, 1983; Tosi *et al.*, 1988). Accumulation in plants is influenced by light (Sütfeld, 1982) and fungal infection (Kourany and Arnason, 1988). The extent to which endogenous processes (*e.g.* synthesis, transport, degradation)

contribute to the observed accumulation patterns has not been studied.

The majority of investigations has been devoted to thiophene accumulation in cell and organ cultures. It was observed that cells cultured in liquid medium, and disorganized (*Agrobacterium*-transformed) calli on solid medium usually accumulate much lower amounts of thiophenes than excised roots cultured *in vitro*. (Norton *et al.*, 1985; Ketel, 1987; Croes *et al.*, 1989[a]). This raised the possibility that differentiation and development of organized tissues (roots) is required for optimal thiophene synthesis. Upon transformation by *Agrobacterium rhizogenes*, transgenic root cultures were obtained which grow rapidly and accumulate large amounts of thiophenes (Flores *et al.*, 1988; Croes *et al.*, 1989[b]; Mukundan and Hjortso, 1990). These roots have become favorite research tools, because of easy culturing procedures, constancy of root morphology, and high thiophene contents.

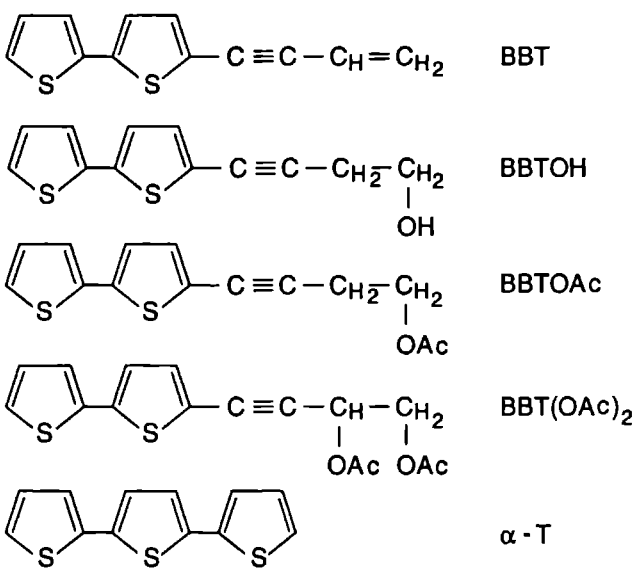

Figure 1.4 Thiophenes of *Tagetes patula* and *T. erecta*.

THIS THESIS

Further development of the *Tagetes*/thiophene system as a model to study the regulation of secondary metabolism requires new investigations in the areas of physiology, metabolism, biochemistry, and cellular and molecular biology. Ultimately, these investigations should lead to the purification of enzymes of thiophene synthesis and the cloning of genes encoding such enzymes. Characterization of the biochemical properties of biosynthetic enzymes and elucidation of the mechanisms that regulate the transcription and translation of the corresponding genes will lead to a comprehensive understanding of the regulation of thiophene metabolism.

In view of the limited knowledge of the *Tagetes*/thiophene system, a straight forward approach towards these goals (enzyme purification and gene cloning) was not feasible at the outset of this project. No single step of thiophene biosynthesis was characterized adequately at the biochemical level. Furthermore, the highly non-polar nature of thiophenes indicated that these compounds are most probably synthesized by membrane-bound enzymes, which are usually resistant to biochemical purification. A direct approach to gene cloning, omitting the enzyme purification step, was also impracticable. Tagged mutants of thiophene metabolism (containing a known transposon or T-DNA sequence in a gene of thiophene metabolism), which could be used for gene cloning, were not available. An alternative approach towards direct gene cloning could involve the differential screening of a cDNA library. However, to be successful this approach requires the availability of otherwise identical tissues that only differ (greatly) by their thiophene synthesizing activity. Such tissues were not available at the start of the project.

The investigations reported in this thesis were aimed at the further development of the *Tagetes*/thiophene system as a versatile model to study the regulation of plant secondary metabolism. The first part of the work provides a detailed physiological analysis of thiophene accumulation during plant development in two *Tagetes* species. In chapter 2, the accumulation of

thiophenes in organs and tissues of young developing *Tagetes* plants is described, and the extent to which synthesis and transport contribute to the observed accumulation patterns is determined. Also, in chapters 2 and 4, data are presented with respect to the localization of thiophenes at the tissue and cellular level. In paralel with these basic investigations, a mutagenesis approach was adopted to study the metabolic pathway of thiophene synthesis in more detail. Chapter 3 describes the isolation and characterization of mutants of thiophene metabolism in *T. erecta*. These mutants were used to elucidate a part of the thiophene pathway. The mutants also provided valuable precursors that could be used to proceed to the next step, which was the characterization of separate conversions of thiophene biosynthesis (Chapter 4). In these experiments, protoplasts were used since they constitute a *semi in vitro* system which may be useful to optimize the conditions for purification of functional enzymes. In another, independent, line of research, transgenic root cultures were used to investigate the activity of thiophene synthesis in relation to sulfate availability (Chapter 5). This work provided information on the activity and regulation of thiophene metabolism in relation to primary metabolism and yielded tissues that were useful to compare expression levels of genes which might be involved in thiophene synthesis. The final part of the investigations (Chapter 6) comprised the construction of a cDNA library from mRNA isolated from transgenic *Tagetes* roots, and the characterization of several cDNA clones from this library. The expression levels of the genes corresponding to selected cDNA clones were related to thiophene synthesis in various plant organs and in cultured roots.

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CHAPTER 2

THIOPHENE SYNTHESIS AND DISTRIBUTION IN YOUNG DEVELOPING PLANTS OF *TAGETES PATULA* AND *TAGETES ERECTA*

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ABSTRACT

Thiophene synthesis and accumulation were investigated in organs of *Tagetes patula* and *T. erecta*. Thiophene accumulation started rapidly in germinating seedlings of both species. Roots and hypocotyls were the major thiophene accumulating organs and BBT and BBTOAc were the major accumulated compounds. Higher thiophene concentrations were reached in *Tagetes patula* than in *T. erecta*, and the accumulation patterns for individual thiophenes were different within organs, between organs and between both species. Within hypocotyls of *Tagetes patula*, thiophene concentrations were high in the epidermis and vascular tissue and low in the parenchymatic tissues of cortex and pith. Synthesis of thiophenes was high in the roots and hypocotyls and very low in the leaves. Transport of thiophenes from the roots into the shoot occurred but the rate of transport was low and could not be responsible for the high concentrations in the hypocotyl. It is concluded that, for the main part, thiophenes are accumulated were they are synthesized.

INTRODUCTION

Though the term "secondary" suggests otherwise, many secondary metabolites are of great importance to the producing organism, since they improve its evolutionary fitness in one way or another (Harborne, 1988). Cultivated plant species produce secondary compounds which either enhance their productivity (by attracting pollinators, repelling or poisoning predators and pathogens) or are themselves valuable products for man (pharmaceuticals, spices, dyes).

Formation of secondary compounds can be accurately quantified and can be easily manipulated in many cases. The enzymes of secondary pathways are encoded by a limited number of genes which can be identified and cloned (Hahlbrock and Scheel, 1989). This opens the way to studies of the regulation of secondary metabolism at the molecular level. Knowledge of the regulatory

processes at the molecular level may be used to manipulate secondary metabolite accumulation in plants to improve their economic value (Van der Krol *et al.*, 1988; Van Tunen and Mol, 1990; Bailey, 1991).

Accumulation of secondary compounds is a strictly regulated process in plants. The establishment of secondary metabolism is usually closely linked to cellular and morphological differentiation and may thus be considered as biochemical differentiation (Wiermann, 1981). The result of this differentiation is that only specialized cells in certain organs synthesize and accumulate specific secondary compounds (Yerger *et al.*, 1992). Synthesis and accumulation are triggered by developmental cues (Wiermann, 1981; Van Tunen and Mol, 1990), but may also be elicited by external signals like fungal cell wall components and UV-light (Kuhn *et al.*, 1984; Schmidt and Ebel, 1987; Lois *et al.*, 1989). The kinetics of net accumulation is determined by the extent of synthesis, transport, storage and breakdown. Regulation may occur in each of these processes by different mechanisms (molecular, enzymic, metabolic) at the cellular, tissue, and organ level.

A prerequisite to studies on the regulation of secondary metabolite production in a species of interest is a precise knowledge of the accumulation kinetics and the distribution patterns of the secondary compound of interest in different organs. Furthermore the contributions of synthesis, transport and degradation to the observed accumulation patterns in different organs must be recognized. This analysis needs to be extended to the tissue level. Finally, genetic factors which may be responsible for differences that exist between species or varieties within a species should be considered.

Tagetes species produce thiophenes (Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1979), polyacetylenic compounds, characterized by one, two, or three aromatic, five-membered, sulfur-containing rings, that are linked together by their α-carbons. The compounds possess a strong nematicidal activity as was first shown by Uhlenbroek and Bijloo (1958, 1959), rendering the producing *Tagetes* plants very useful for suppressing nematode populations in the soil. The compounds are also toxic to bacteria and fungi (Chan *et al.*, 1975) and insects

(Green *et al.*, 1991), and even anti-HIV activity has been reported (Hudson *et al.*, 1993). Their toxicity is strongly enhanced by near UV-light (Gommers and Bakker, 1988).

The major thiophenes in *T. patula*, BBT and BBTOAc are mainly accumulated in roots and stems whereas low amounts of BBTOH and α-T are present in all organs (Fig. 2.1; Sütfeld, 1982; Downum and Towers, 1983; Tosi *et al.*, 1988). The reports disagree considerably upon absolute concentrations and accumulation curves of thiophenes in *T. patula*. Data on accumulation and distribution of thiophenes in *T. erecta* are scarce and indicate that thiophene concentrations in this species are lower than in *T. patula* (Croes, 1989[a]; Mukundan and Hjortso, 1990).

No attempts have been made until now to establish a relationship between accumulation and synthesis of thiophenes. Roots are capable of autonomous thiophene synthesis as is evident from the high thiophene concentrations of *in vitro* grown detached roots (Croes *et al.*, 1989[b]; Mukundan and Hjortso, 1990) and from the observation that thiophene accumulation does not start until roots begin to emerge on calli (Croes *et al.*, 1989[a]). That thiophene concentrations in detached roots are higher than in attached roots, opens the possibility that in intact plants thiophenes are transported into the shoots.

Almost nothing is known about the localization of thiophenes at the tissue level. Downum and Towers (1983) and Caniato *et al.* (1990) reported the presence of thiophenes in glandular trichomes and secretory glands of leaves of *T. patula* but they gave no indication of the concentrations. Van Fleet (1972) claimed that thiophenes are concentrated in the endodermis of roots of marigolds which was weakly supported by sulfur distribution patterns in roots of *T. patula* (Makjanic et al. 1988).

In this paper, new data are presented concerning the kinetics of thiophene accumulation in different organs of *Tagetes* plants during development. Contributions of synthesis and transport to the observed accumulation patterns are evaluated. Also the localization of thiophenes in hypocotyl tissues is studied in detail. For the first time different cultivars of *T. patula* and *T. erecta* were employed in one investigation.

CHAPTER 2

MATERIALS AND METHODS

Plant growth

Plants of *Tagetes erecta* and *T. patula* were grown in a growth chamber at 25 °C and 70% relative humidity under a 16-hour photoperiod in 50-ml pots containing moist vermiculite. Half-strength Hoagland solution was administered to the plants every second day. Plants that were used for hypocotyl dissection were grown in soil during the summer in a greenhouse under natural light.

Hypocotyl dissection

Hypocotyls of six-weeks-old soil-grown plants were manually dissected under a binocular dissecting microscope. The epidermis was peeled off with tweezers; other tissues were separated using a razor blade. Tissues were kept in a humid environment to prevent desiccation, and were weighed and stored at -20 °C (submerged in 1 ml ethanol) as soon as possible. Correct separation of tissue types was verified by microscopic examination of cross sections of hypocotyls at all stages of dissection.

Thiophene analysis

Dry seeds and seeds that had been allowed to germinate for one day (*T. patula*) or one and two days (*T. erecta*) were extracted as "whole organisms". Thereafter roots, hypocotyls, cotelydons, stem segments, and leaves were extracted separately. On days zero (dry seeds) to six, tissues were pooled from three to six seedlings per sample and three samples were extracted each day. On days 7, 14, 21 and 28, organs from at least five individual plants were separately extracted. Thiophenes were extracted and analyzed as described before (Croes, 1989[b]). Briefly, tissue samples were ground in ethanol: water (1:1 v/v) and thiophenes were partitioned into a mixture of hexane: *t*-butylmethylether (1:1 v/v). The organic solvents were evaporated under nitrogen gas and the thiophenes were recovered in a small volume of ethanol. The extracts were subjected to HPLC analysis on a Lichrosorb RP-18 column and the thiophenes in the eluate were quantified on the basis of UV absorption at 340 nm.

Preparation of radioactive thiophenes

Radioactive thiophenes were obtained by incubating young plants for several days to one week in growth medium containing 15 MBq of ^{35}S-labeled sulfate (specific activity 750 MBq.mmol^{-1}) per plant. Thiophenes were extracted as described and purified by subjecting highly concentrated extracts to preparative HPLC on a Lichrosorb RP-18 column with acetonitrile:water (65:35) as the eluent. Selected fractions were pooled, the eluent was evaporated under nitrogen gas and the purified thiophenes were dissolved in ethanol. Purity of the isolated compounds was confirmed by analytical HPLC.

Thiophene synthesis and translocation

The vermiculite was washed off the roots of three-weeks-old plants. These plants were transferred to test tubes containing either 5 ml sulfate-free half- strenght Hoagland solution to which a tracer amount of radioactive sulfate (1.67 MBq/plant) was added or 5 ml complete half-strength Hoagland solution to which one of four radio-active thiophenes (BBTOAc, 4.33 kBq per plant; BBT, 5.25 kBq per plant; BPT, 2.00 kBq per plant; MeBBT, 2.42 kBq per plant) was added. Only the roots were in contact with the growth medium. Plants were harvested at intervals and thiophenes were extracted from roots, hypocotyls, and leaves and analyzed by HPLC. Radioactivity in thiophenes and other apolar compounds was determined, by collecting the eluate of HPLC runs in 75 fractions of 0.5 ml per run, and counting the radioactivity in all fractions in a liquid scintillation counter. From one set of plants the roots were removed prior to incubation in the ^{35}S-sulfate-containing solution.

RESULTS

Thiophenes in roots of five Tagetes varieties

To evaluate cultivar-dependent differences in thiophene accumulation, thiophene concentrations were determined in the roots of four-weeks-old maturing plants of four commercial cultivars of *T. patula* and *T. erecta* and of *T. erecta* plants from the Botanical Garden of the University of Nijmegen.

Thiophene concentrations were five to seven times higher in roots of *T. patula* than in roots of *T. erecta*, irrespective of the variety (Table 2.1). The three cultivars of *T. erecta* considerably differed in growth rate (mg FW day^{-1}) and thiophene content, but there was no relation between these variables. BBT and BBTOAc were the major thiophenes in both species. In addition, small amounts of BBTOH, $BBT(OAc)_2$ and α-T were found in all varieties. In the roots of *T. erecta* the ratio BBTOAc/BBT was consistently higher than in *T. patula*. Chemical structures of all thiophenes detected are presented in Fig 2 1.

Table 2.1 Root fresh weight (FW), thiophene concentration, total thiophene amount, and contribution of different compounds to thiophene content in roots of five *Tagetes* varieties at the age of four weeks Data are the means of determinations on ten individual plants ± s e

cultivar	root FW (mg)	thiophene concn (nmol g^{-1})	total thiophene (nmol)	BBT (%)	BBTOAc (%)	other (%)
T pat cv nana	510 8 ± 42 6	1466 0 ± 198 8	725 0 ± 72 5	85	11	4
T pat cv sparky	527 0 ± 32 6	910 6 ± 67 6	479 9 ± 47 8	77	21	2
T er L	798 7 ± 39 1	135 9 ± 11 5	110 1 ± 13 5	65	32	3
T er cv cupid orange	470 1 ± 65 2	207 9 ± 17 1	97 8 ± 15 0	46	49	5
T er cv cracker jack	1001 4 ± 127 5	182 3 ± 28 9	185 4 ± 28 9	33	63	4

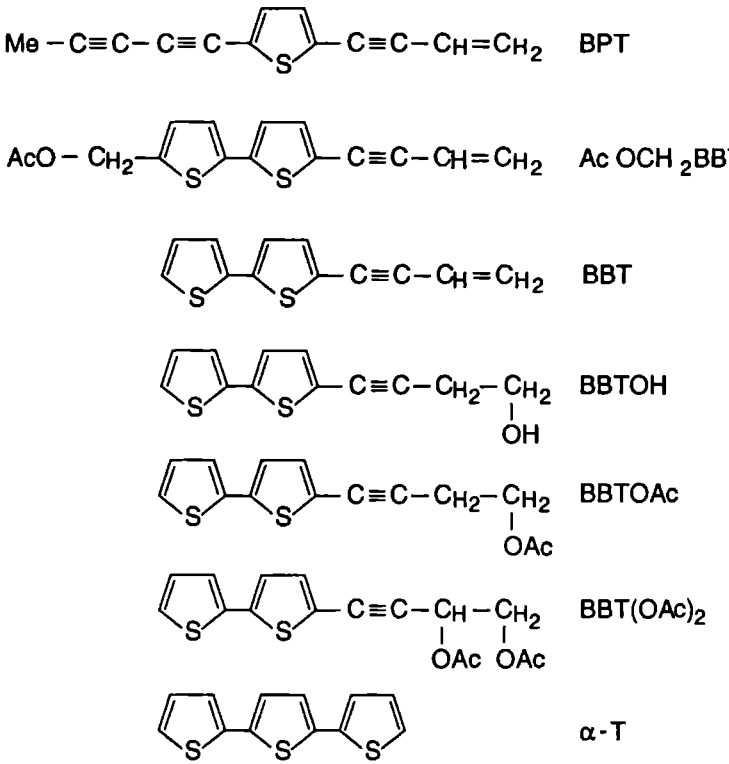
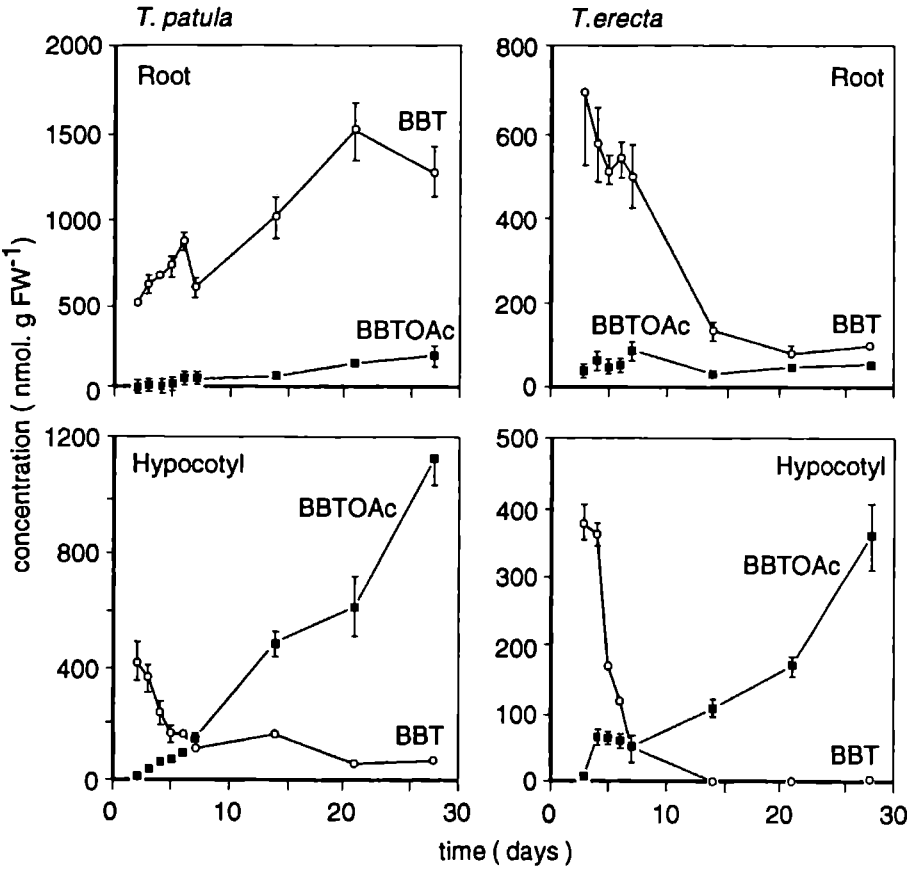


Figure 2.1 Chemical structures of thiophenes in *Tagetes patula* and *T. erecta*.

Thiophene distribution in T. patula and T. erecta

The large difference in thiophene content in roots of four-weeks-old *T. erecta* and *T. patula* indicated that the accumulation kinetics must be different in the two species. It also raised the question of how thiophene concentrations compared in other organs of these plants. To address these questions, the concentrations of individual thiophenes were recorded during a four-weeks-period in different organs of developing *T. erecta* from the Nijmegen Botanical Garden and *T. patula* cv. Nana, which represented the extremes of thiophene concentrations in the primary screen.

BBT and BBTOAc were the major thiophenes in roots and hypocotyls of both species during the entire period of investigation (Fig. 2.2). Small amounts of BBTOH, $BBT(OAc)_2$, $AcOCH_2BBT$ and α-T found in the roots of *T. patula* accounted for no more than 4 percent of the thiophene content in the roots at any time (data not shown). In the stems of plants of both species a gradient was observed, with the thiophene concentration decreasing from the base to the apex (Table 2.2). Minor thiophenes were relatively more prominent in hypocotyls than in roots, their contribution to the total content increasing towards the apex. Rather high concentrations of the monothiophene BPT were found in the stem of *T. patula* (Table 2.2).



Since thiophene concentrations were higher in organs of *T. patula*, it was easier to identify minor compounds in this species. The concentrations of all identified thiophenes in various organs of *T. patula* and in roots and hypocotyls of *T. erecta* at the age of two weeks are presented in Table 2.2.

Table 2.2 Thiophene concentrations in organs of two-weeks-old plants of *Tagetes patula* and *T. erecta*.

Data are the means of determinations on five individual plants.

T. patula	thiophenes (nmol.g FW^{-1})					
	$BBT(OAc)_2$	BBTOAc	$AcOCH_2BBT$	BBT	BPT	α-T
root	8.5	61.2	11.7	1004.4	0.0	10.0
hypocotyl	13.4	478.1	17.0	156.9	19.1	13.8
1st internode	6.4	346.5	24.1	37.3	130.9	77.6
cotyledon	0.0	1.7	25.0	1.1	0.0	0.0
leaf	0.0	2.6	17.9	2.2	1.2	0.0
T. erecta						
root	3.2	24.2	0.0	127.5	0.0	0.0
hypocotyl	3.9	105.0	0.0	0.0	0.0	0.0

Figure 2.2 Concentrations of BBT (o) and BBTOAc (≡) in roots and hypocotyls of *T. patula* and *T. erecta* during plant development. Roots and hypocotyls were analyzed separately from 2 days (*T. patula*) or 3 days (*T. erecta*) of germination onwards. Data are the means of determinations on three samples of pooled plant organs (day 0 to 6) or five individual plants (day 14, 21, 28) ± se.

Cotyledons and leaves of young plants of both species contained only small amounts of thiophenes. The total thiophene concentration in these organs during the first four weeks of plant growth was at all times less than 5 % of the concentration in roots (data not shown). The major thiophene in cotyledons and leaves of both species was $AcOCH_2BBT$.

In germinating seeds and in developing roots and hypocotyls of both species, accumulation of BBT started very soon upon the onset of germination and the concentration rose rapidly during the first few days of seedling development (Table 2.3; Fig 2.2) In hypocotyls of both species and in roots of *T. erecta* an early peak was reached after three to four days, followed by a gradual decline of BBT concentration during the rest of the growth period. Only in roots of *T. patula* was the early peak followed by a further gradual increase in BBT concentration (Fig. 2.2). BBTOAc was detectable in both species from two days of imbibing onwards (Table 2.3). Its concentration gradually rose to high values in hypocotyls of both species, but remained relatively low in the roots throughout the period investigated (Fig. 2 2).

Table 2.3 Thiophene concentrations in germinating seeds of *T patula* and *T erecta* Data are the means of determinations on three samples ± se

germination (days)	thiophenes (nmol g FW^{-1})			
	T patula		*T erecta*	
	BBTOAc	BBT	BBTOAc	BBT
0	0 0 ± 0 0	0 0 ± 0 0	0 0 ± 0 0	0 0 ± 0 0
1	0.0 ± 0 0	182 1 ± 4 7	0 0 ± 0 0	63 7 ± 7 8
2	9 5 ± 2 2	465 9 ± 45 2	16 1 ± 2 8	422 4 ± 64 0
3	28 6 ± 4 4	489 9 ± 38 9	18 7 ± 4 4	464 8 ± 57 4

Tissue localization

Distribution of thiophenes within an organ was investigated in hypocotyls of young mature soil-grown *T. patula* at the age of six weeks. These hypocotyls could be separated into a layer of epidermal plus adhering disrupted sub-epidermal cells; a layer of soft parenchymatic tissue from the cortex; a layer of vascular tissue and the parenchymatic central pith. Much higher thiophene concentrations were found in the epidermal layer and vascular tissue than in the parenchymatic tissue layers (Fig. 2.3). The thiophene concentration in the cortex was one third of that in the epidermis, and in the pith it was only ten percent of that in the vascular tissue. In all tissues BBTOAc and BBT were the major thiophenes, but the ratio between these compounds changed in favour of BBT from the outside to the core. Thiophene concentrations in whole hypocotyls that were calculated from values of the separated tissues (BBTOAc: 136 nmol.g FW^{-1}; BBT: 27 nmol.g FW^{-1}) agreed well with measured concentrations in intact hypocotyls (BBTOAc: 123 nmol.g FW^{-1}; BBT: 32 nmol.g FW^{-1}), which indicated that dissection had not affected recovery of thiophenes.

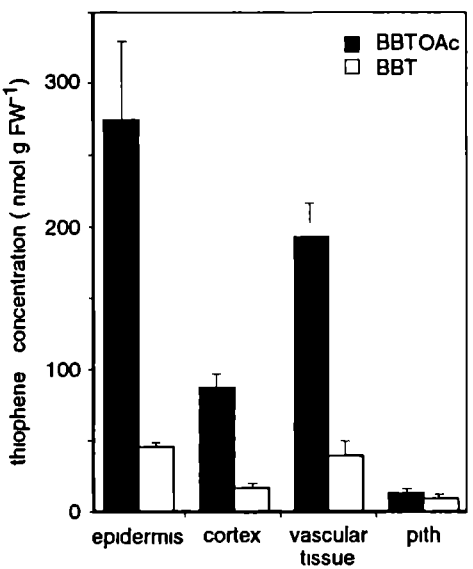


Figure 2.3 Concentrations of BBTOAc (closed bars) and BBT (open bars) in tissues of dissected hypocotyls of soil-grown *T. patula* at the age of six weeks. Data are the means of separate determinations on tissues of five individual plants ± se.

Thiophene synthesis

Active synthesis of thiophenes in roots, hypocotyls, and leaves of *Tagetes* plants at the age of three weeks was monitored by adding radioactive sulfate to the growth medium and determining the incorporation of ^{35}S-sulfur into thiophenes in the organs at time intervals. The capacity of aerial plant parts to synthesize thiophenes independently of roots, was assessed by incubating detached shoots in ^{35}S-sulfate-containing medium.

After 44 hours of incubation in radioactive sulfate-containing growth medium, plants of *T. patula* and *T. erecta* had absorbed 85% and 97%, respectively, of the radioactivity added. Figure 2.4 illustrates the rapid spreading of radioactive sulfate into roots, hypocotyls and leaves of intact *T. patula* during a 44-hour uptake period and its subsequent partitioning into thiophenes (BBT and BBTOAc) and minor unidentified apolar compounds. In roots and hypocotyls radioactivity was rapidly incorporated into BBT and BBTOAc. In leaves only a low percentage of radioactivity was incorporated into unidentified sulfur-containing apolar compounds that eluted early from the HPLC column relative to BBTOAc and BBT. In *T. erecta* spreading of radioactive sulfate into the organs was equally fast, but the rate of incorporation into thiophenes was about three times lower than in *T. patula*. Also in hypocotyls of detached shoots of both species a significant percentage of radioactivity was incorporated into thiophenes after a 44-hour incubation in radioactive sulfate (3.8 ± 0.3 % and 2.3 ± 0.2 % for *T. patula* and *T. erecta*, respectively). BBT and BBTOAc were synthesized in the hypocotyls of these detached shoots in the same ratios as in hypocotyls of intact plants, which indicates that no changes in synthetic activities had occurred in these hypocotyls within 44 hours of detachment from the roots.

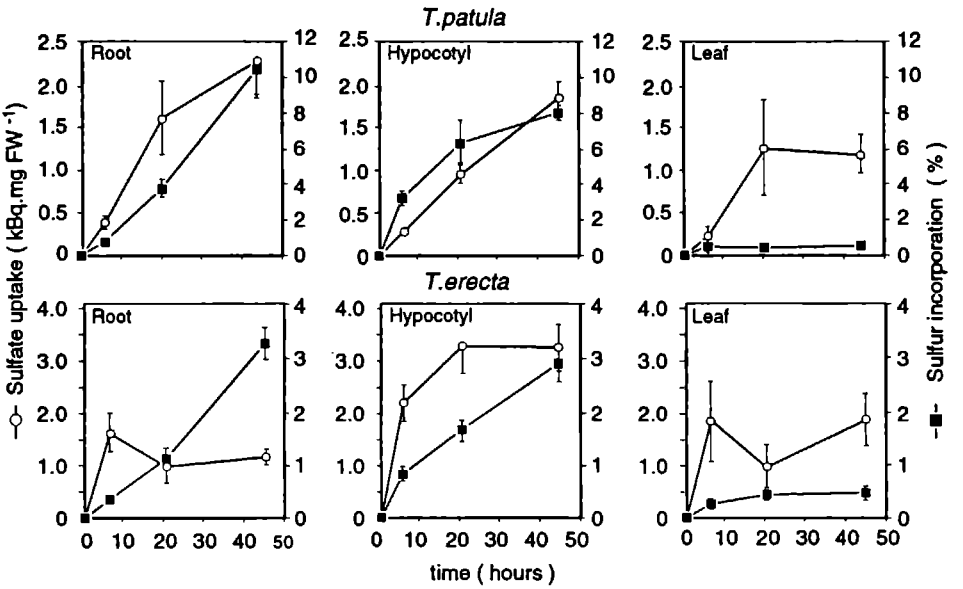


Figure 2.4 Uptake of ^{35}S-sulfate (o) into roots, hypocotyls and leaves of *T. patula* and *T. erecta*, and partitioning into thiophenes (=), during a 44-hour incubation in ^{35}S-sulfate-containing growth medium. Data are averages of determinations on four plants ± se.

The importance of thiophene transport was assessed by incubating plants in growth medium containing radioactive thiophenes. When intact plants of *T. erecta* were placed in growth medium supplemented with ^{35}S-labeled thiophenes, over 90 % of the radioactivity was taken up by the plants within 44 hours (Table 2.4). However, only 4 to 10 % of the radioactive thiophenes were translocated into the hypocotyls after this period and no thiophenes were found in higher plant parts. In contrast, when radioactive sulfate was fed, equal amounts of radioactive thiophene were found in roots and hypocotyls after 44 hours (Table 2.4).

Table 2.4 Uptake of ^{35}S-sulfate and ^{35}S-thiophenes from the medium by *Tagetes erecta* plants, and radioactivity in thiophenes in roots and hypocotyls, after 44 hours of incubation with the radioactive compounds.

Data are the means of determinations on four (^{35}S-sulfate) or two (^{35}S-thiophenes) plants ± se.

^{35}S-labeled compound added to medium	radioactivity taken up (%)	thiophene-associated radioactivity			
		roots (Bq)	hypocotyls (Bq)	roots (%)	hypocotyls (%)
sulfate	97.5 ± 1.1	10640 ± 1062	10051 ± 1230	52 ± 5	48 ± 5
BBTOAC	85.1 ± 8.3	698 ± 21	73 ± 42	91 ± 5	9 ± 5
BBT	95.2 ± 4.0	1064 ± 10	90 ± 14	92 ± 1	8 ± 1
BPT	94.0 ± 0.1	549 ± 60	21 ± 9	96 ± 2	4 ± 2
MeBBT	91.7 ± 0.3	603 ± 229	33 ± 14	93 ± 5	7 ± 5

DISCUSSION

Thiophenes are very unevenly distributed over the organs of *Tagetes* plants and over the tissues within an organ. In *T. erecta* and *T. patula* the major thiophenes (BBT and BBTOAc) accumulate mainly in roots and the lower part of the stem. Other thiophenes are found at low concentrations and only low thiophene concentrations are found in leaves. Within hypocotyls, thiophenes are concentrated in the epidermis and in the vascular tissue. Among cultivars of both species, relatively small differences of thiophene concentration in roots are observed compared to the differences between the species. The major thiophenes, BBT and BBTOAc, display characteristic, distinctive accumulation patterns in the roots and hypocotyls of *T. patula* and *T. erecta* during plant development. Much higher thiophene concentrations are achieved in *T. patula* than in *T. erecta*. This is not related to the growth rate of both species, nor to the rate of uptake and distribution of sulfate. Rather it is due to a higher rate of thiophene synthesis in roots and hypocotyls of *T. patula* compared to *T. erecta*.

Active biosynthesis of thiophenes takes place in the organs were they accumulate, and transport of thiophenes from roots into hypocotyls is of limited importance.

The large differences of thiophene content in *T. patula* and *T. erecta* cannot be accounted for by their growth rates (Table 2.1). The thiophene concentration in one *erecta* cultivar, the growth rate of which is comparable to that of both *patula* cultivars, is five to seven times lower than it is in the *patula* cultivars. Among the *erecta* cultivars, growth rate and thiophene concentration are not correlated. Thus the thiophene concentration seems to be a genetically determined trait of both species, which is not strongly related to other traits (*e.g.* growth rate) of individual cultivars.

Our work shows that accumulation of the individual thiophenes is differentially regulated within organs, between organs, and between two species (Fig. 2.2). The accumulation patterns of BBT and BBTOAc are different in roots and hypocotyls and the concentrations of these compounds even change in opposite directions in hypocotyls. Furthermore, in roots of one species (*T. erecta*) the concentration of BBT declines after an early peak whereas in roots of the other species (*T. patula*) the concentration keeps increasing, resulting in a much higher BBT level in roots of *T. patula*. The total amount of BBT keeps increasing in roots of both species, indicating that in both species active BBT synthesis occurs throughout the period investigated, albeit at greatly differing rates.

The precursor feeding experiments with ^{35}S-labelled sulfate and thiophenes to intact plants and detached shoots show that thiophenes are being synthesized in the organs where they accumulate: roots and hypocotyls (Fig. 2.4). Uptake and distribution of ^{35}S-sulfate is rapid in both species, but in *T. patula* the rate of incorporation into thiophenes is three times higher. This points towards a higher synthetic activity in *T. patula* than in *T. erecta*, which, assuming that breakdown is negligible (Arroo *et al.*, 1990), is the main reason for the higher thiophene concentrations in *T. patula*. The low thiophene content of leaves is not due to limited supply of precursor sulfate to the leaves but

rather to a very low synthetic activity in leaves. Thiophenes can be transported from roots into above-ground parts, but the rates of transport are low and cannot explain the high thiophene concentrations in hypocotyls (Table 2.4).

Thiophenes are strictly localized, not only at the organ level, but also in tissues of an organ. This is exemplified by the hypocotyl (Fig. 2.3), in which thiophene accumulation is most pronounced in the epidermis and the vascular tissue, sites from which the compounds do not diffuse extensively to surrounding tissues. In the context of the functioning of thiophenes as defensive agents, localization in the epidermis and the vascular tissue makes sense, since these tissues are most and first exposed to foreign intruders.

Obviously, seedlings are extremely vulnerable when attacked by pathogens or predators. Therefore, the rapid establishment of high concentrations of biocidal thiophenes that occurs in seedlings of both species, may be imperative for their survival. The timing of maximum BBT concentration in hypocotyls of both species (on days two-three and days three-four for *T. patula* and *T. erecta*, respectively), coincides with emergence of the hypocotyls above the vermiculite substrate. This suggests that emergence triggers a regulatory change. The reason for this change may be that the most vulnerable period has ended, and that therefore BBT concentration may drop. Alternatively emergence implies exposure to light, which highly increases the toxicity of thiophenes. Therefore, it may be necessary for the plant to reduce its thiophene level at this stage to prevent self-toxication. Thirdly, since thiophenes are unstable in the light, the concentration may drop due to photo-degradation.

Previous investigations in which different *Tagetes* cultivars were used, led to considerably varying estimates of thiophene concentrations (Downum and Towers, 1983; Tosi *et al.*, 1988; Croes *et al.*, 1989[a]; Croes *et al.*, 1989[b]; Mukundan and Hjortso, 1990). Our comparison of various cultivars suggests that the differences found by these authors cannot simply be attributed to the use of different plant material. Possibly, culturing conditions and seed quality have an influence on seedling development and thereby on kinetics of thiophene accumulation. It is therefore important to standardize these conditions. The

accumulation patterns that we observed were more complex than those reported by others (Sütfeld, 1982; Downum and Towers, 1983; Tosi *et al.*, 1988). Their results suggested that BBT and BBTOAc were synthesized in more or less fixed proportions at all times, and that regulation of thiophene synthesis was similar in roots and hypocotyls.

The results presented in this chapter contribute importantly to the knowledge of thiophene accumulation in *Tagetes*. Profiles of thiophene accumulation in different organs during plant development have been determined, and the localization of thiophenes at the tissue level, as well as aspects of thiophene synthesis and transport have been clarified. This information will be very usefull for further investigations in various directions. The most important result is that not only roots, but also hypocotyls are very active in thiophene synthesis and accumulation, and may therefore be employed as an alternative source of biological starting material. In one line of future research described in this thesis (Chapter 4), hypocotyl derived protoplasts will be used to study the cellular localization of thiophenes and the biosynthetic capacities of isolated cells.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF MUTANTS OF THIOPHENE SYNTHESIS IN *TAGETES ERECTA*

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ABSTRACT

Two mutants of *Tagetes erecta* displaying aberrant thiophene composition were identified by screening over 300 plants from a mutagenized M2 population using HPLC analysis of root extracts. Both mutants, which may have originated from the same mutational event, contained high amounts of the C_{13} monothiophene BPT that was previously not found in T. erecta, and also high amounts of two C_{13} bithienyls which were absent or present at low concentrations in the wild type. The mutant phenotype was also expressed in 21 *Agrobacterium rhizogenes* transformed root clones derived from both mutants. Feeding experiments with root cultures derived from one mutant and from the wild type, indicated that the monothiophene accumulating in the mutant is the common precursor for all bithienyl thiophenes in wild-type and mutant *Tagetes erecta*. These experiments also showed that the tested mutant is deficient in demethylation of the monothiophene.

INTRODUCTION

Although mutants have been widely applied to investigate metabolic pathways in micro-organisms and fungi, they have been used relatively rarely for this purpose in plants. Most metabolic mutants of plants concern primary metabolic processes, like carbon assimilation (Somerville & Ogren, 1979), metabolism of starch (Caspar *et al.*, 1985), lipids (Browse *et al.*, 1985; Kunst *et al.*, 1989) or amino acids (Haughn & Somerville, 1986; Kreps and Town, 1991;Frankard *et al.*, 1992; Wright *et al.*, 1992; Wu & King, 1994), whereas only few mutants of secondary metabolic pathways are known (Haughn *et al.* 1991; Chapple *et al.* 1992; Rathjen & Robinson, 1992). This situation exists in spite of the fact that chemically induced loss-of-function mutations can relatively easily be obtained (Haughn & Somerville, 1987).

Tagetes species (marigolds) produce, mainly in the roots, aromatic sulfur-containing compounds known as thiophenes, which are toxic to nematodes when ingested (Uhlenbroek and Bijloo, 1958; 1959). Co-occurrence patterns and precursor feeding experiments in related species have led to a generally accepted biogenetic scheme, as depicted in Figure 3.1 (Bohlmann *et al.*, 1973; Bohlmann *et al.*, 1985). Oleic acid is converted into tridecapentaynene (PYE) via repeated steps of desaturation and chain shortening. PYE is then converted into the thiophenes that accumulate in *Tagetes* species (Bohlmann & Berger, 1965; Bohlmann & Hinz, 1965; Schulte *et al.*, 1965; Bohlmann *et al.*, 1966). The key step in this conversion is the addition of H_2S or its biochemical equivalent to conjugated triple bonds and subsequent ring formation, which is probably a two-step reaction (Bohlmann *et al.*, 1973). In addition to the formation of two

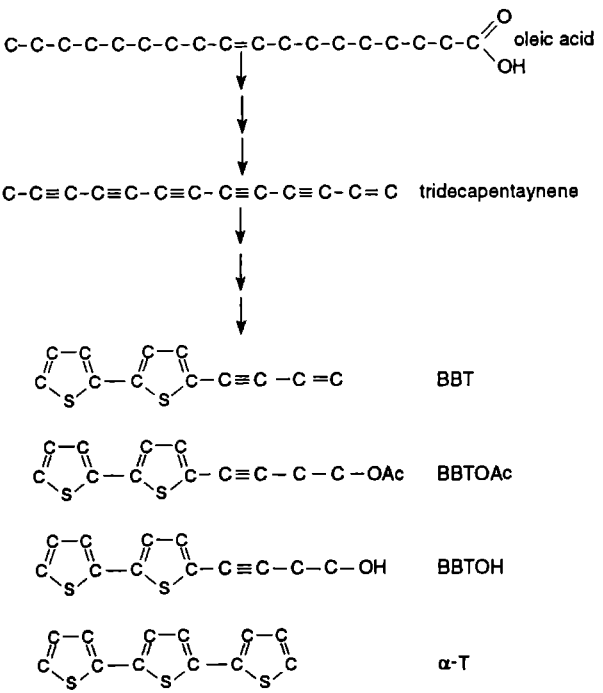

Figure 3.1 Proposed pathway for thiophene biosynthesis in *Tagetes* species.

or three thiophene rings, also removal of a terminal methyl group and modification of a vinyl group are necessary to obtain the various thiophenes that finally accumulate (Fig. 3.1).

Not much is known about the order in which these reactions take place in *Tagetes*, nor about the specificity of the enzymes involved. Some of the postulated intermediates, like PYE and the monothiophene, had not been identified in *Tagetes* when this work was started, and none of the presumed enzymes have been characterized biochemically. Indeed, it is not even sure whether thiophene ring formation requires an enzyme activity, since this reaction has been reported to proceed spontaneously under certain circumstances (Schulte *et al.*, 1962).

In view of the many open questions concerning thiophene biosynthesis, it was decided to follow a genetic approach, involving the generation of mutants, to study thiophene synthesis in *Tagetes*. To induce mutations, the chemical chemical mutagen EMS was applied to seeds, which resulted in chimeric M1 plants. After self-pollination of these plants, populations of M2 plants were obtained, which were screened for desired phenotypes. Since interest was primarily directed towards mutants of the late steps of thiophene synthesis (from ring formation onwards), a screening procedure was used in which root extracts from M2 plants were separated by HPLC and the eluent was analyzed at a wavelength suitable for thiophene detection. In this way mutants should be identified that either over- or under-produce known thiophenes or that produce thiophenes unknown from wild-type plants. *Tagetes erecta* L., a self-fertilizing diploid species (2n = 24) for which no genetic studies have been described, was used in the experiments.

In this chapter, the isolation is reported of mutants of *T. erecta* that produce a different spectrum of thiophenes compared to the wild type. Precursor feeding experiments with mutant plants and hairy root cultures derived from them provided important clues with regard to the biochemical defect of the mutants and also yielded important new information concerning thiophene biogenesis in *Tagetes erecta*.

CHAPTER 3

MATERIAL AND METHODS

Mutagenesis

Seeds of *Tagetes erecta* L. were from the Botanical Garden of the University of Nijmegen. Batches of seeds were treated with vigorously stirred aqueous solutions of EMS at various concentrations 0 1, 0.2, 0 4 or 0.8 % EMS during 16 hours (Exp 1), and 0 8, 1.6 or 3 2 % EMS during 8 hours or 6.4 % EMS during 4 hours (Exp. 2). At the end of the treatment seeds were thoroughly washed for several hours in running tap water

Each treatment was given to four batches of 30 seeds. Germination was scored in all batches. M1 plants were grown from one batch of seeds in each treatment. Growth of the plantlets was analyzed and color variegation of the leaves was scored After 20 to 25 days, plantlets were transferred to soil in the greenhouse. After 14 to 18 weeks flowers were collected over a 6-week period All flowers from M1 plants that had received the same mutagenic treatment, were pooled.

M2 plants were grown from seeds of M1 plants that had received the 0.8% EMS treatment during 8 hours. Root samples were taken three weeks after sowing from 338 M2 plants which originated from 141 M1 flowers, and these plants were subsequently transferred to soil in the greenhouse. Thiophenes were extracted from the samples and the extracts were analyzed by HPLC. Thiophenes were also extracted and analyzed from 31 control plants Seeds were harvested from M2 plants that were selected on the basis of an altered thiophene profile.

Plant growth

Plants to be used for screening purposes were grown in a growth chamber with a 16-hour photoperiod at 25 °C, in 50-ml pots containing moist vermiculite and two grains of osmocote (Grace Sierra Int.) per pot, until harvest or transfer to soil in the greenhouse. The plants were watered every second day. In all other experiments, plants were grown under the same conditions, but the osmocote grains were omitted and half-strength Hoagland solution was administered every second day.

Establishment and maintenance of hairy root cultures

Transgenic hairy root lines were obtained by transformation of *in vitro* grown wild-type and M3 seedlings of *Tagetes erecta* with *Agrobacterium rhizogenes* strain LBA 9402 (pRi 1855) as

previously described (Croes *et al.* 1989[b]). Transformation of the roots was verified by the ability to synthesize agropine and mannopine (not shown). Transformation of the root clones that were used in precursor feeding experiments was further confirmed by Southern blot hybridization using the radiolabeled Eco RI 15 fragment of the Ri plasmid 1855 as a probe (Birot *et al.*, 1987; results not shown).

Hairy root cultures were maintained in the dark at 25 °C on a rotary shaker at 100 rpm in 50-ml erlenmeyer flasks, containing 20 ml Gamborg's B5 medium (Gamborg, 1970) supplemented with 30 g l^{-1} sucrose and 100 $\mu g.l^{-1}$ biotin. Hairy roots were also maintained in Petri dishes, containing the same medium, solidified with 0 2 % (w/v) Gelrite.

Thiophene analysis

Thiophenes were extracted and analyzed as described before (Croes *et al.*, 1989[a]). Briefly, tissue samples were ground in ethanol water (1:1 v/v) and thiophenes were partitioned into a mixture of hexane.*tert*-butylmethylether (1.1, v/v). The organic solvents were evaporated under nitrogen gas and the thiophenes were taken up in a small volume of ethanol. The extracts were subjected to HPLC on a RP-18 column and the thiophenes in the eluate were quantified on the basis of UV absorption at 340 nm. When radioactive extracts were analyzed, the eluate was collected in 75 fractions per run of 25 minutes and radioactivity in all fractions was measured using a liquid scintillation counter.

Purification and identification of thiophenes

Highly concentrated purified thiophenes were obtained by subjecting concentrated extracts to preparative HPLC on a Lichrosorb RP-18 column (particle size 7 μm; column dimension 25 x 0.4 cm) with acetonitrile.water (70:30) as an eluent. Selected fractions were pooled, the eluent was evaporated under nitrogen gas and the purified thiophenes were dissolved in ethanol. Purity of the isolated compounds was confirmed by analytical HPLC and GC. The compounds were identified using GC/MS and ^{1}H-NMR analysis.

For GC/MS analysis, a capillary column (fused silica WCOT, coated with CP-Sil 5CB, 25m x 0 32mm ID) was used to fractionate the samples. Carrier gas was He, and the flow rate 1.5 ml min^{-1}. Samples of 1 μl were injected at an injection port temperature of 150 °C. The initial oven temperature was 100 °C, the temperature was raised at 15 °C.min^{-1} to 250 °C. The electron impact method (EI) was used to ionize the fractions. Recorded spectra were compared to spectra known from the literature (Bicchi *et al* , 1992; Bohlmann *et al.*, 1964;

Bohlmann *et al.*, 1973; Caniato *et al.*, 1990; Groneman *et al.*, 1984).

Fourier-transformed ^{1}H NMR spectra were recorded on a spectrometer operating at 400 MHz. Samples were measured in $CDCl_3$ with tetramethylsilane as an internal standard. NMR-spectra were compared to spectra known from the literature (Atkinson *et al.*, 1964; Bohlmann & Kleine, 1963; Bohlmann *et al* , 1964; Bohlmann *et al.*, 1965; Bohlmann & Berger, 1965; Bohlmann & Zdero, 1985).

Feeding experiments

Radioactive (^{35}S-labeled) thiophenes were obtained by incubating hairy roots or young plants for several days to one week in growth medium containing [^{35}S]sulfate (10 MBq.ml^{-1}). Radioactive thiophenes were extracted and purified as described above.

In feeding experiments, radioactive thiophenes dissolved in ethanol were added to the culture medium of young hairy root cultures and young plantlets (BPT: 18 nmol of 150 Bq.nmol^{-1} per incubation; MeBBT: 7 nmol of 400 Bq.nmol^{-1} per incubation). The final ethanol concentration never exceeded 0.1 %. Tween-20 was added to a final concentration of 0 1 % After further culturing of the hairy roots or plants for various times, they were rinsed with fresh culture medium and the thiophenes were extracted and analyzed as described above.

RESULTS

Mutant selection

Batches of seeds were treated with various EMS concentrations as described in Material and Methods. M1 plants were grown from seeds of the different treatments and flowers were collected from the M1 plants and pooled per treatment. To assess the effects of mutagenesis, small M2 populations were raised from 30 to 60 seeds of six randomly picked M1 flowers of each pool of flowers.

The appearance of colored sectors on M1 plants and of aberrant seedlings among M2 plants (white, yellow or light-green cotelydons) were good markers for the efficiency of mutation induction. On basis of the observations made on M1 plants and on the pilot populations of M2 plants, the treatment with 0.8 % EMS during 8 hours was chosen for selection of mutants with impaired thiophene metabolism. This treatment hardly affected seed germination and seedling growth in M1 and M2, whereas at the same time variegated M1 plants and mutant M2 plants were observed at a high frequency (68% and 46% of M1 and M2 plants, respectively). A large number of flowers was available from M1 plants that had received this treatment.

Root extracts of 338 M2 plants were analyzed by HPLC. In the extracts of 10 M2 plants, originating from two M1 flowers, two compounds were detected that gave rise to very prominent peaks in the HPLC chromatograms which were not present in extracts from wild-type plants. One of these compounds (initially designated compound "no. 11") was much more abundant than the other one ("no. 13"), as judged from the UV absorption. In addition, a third unknown compound ("no. 7") was detected at much higher concentrations in the mutants than in wild-type plants. HPLC chromatograms of mutant and control root extracts are presented in Figure 3.2.

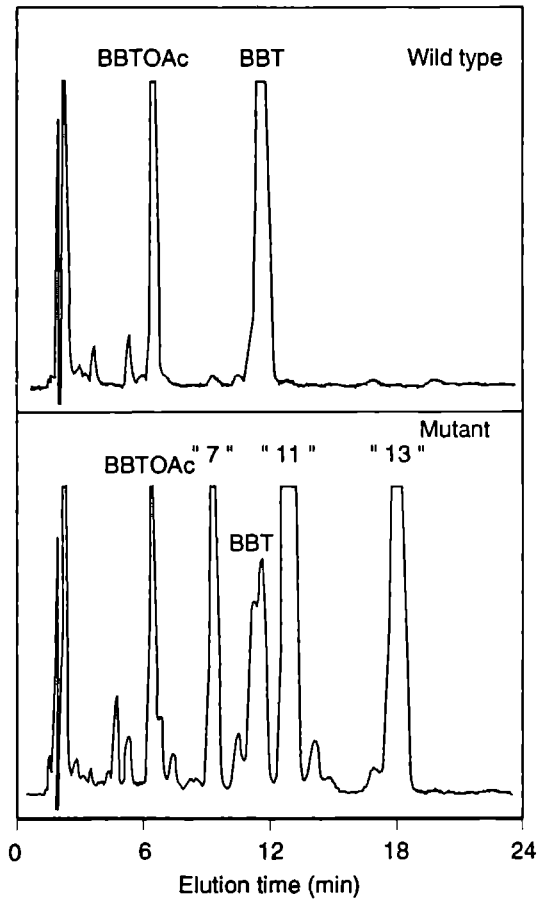

Figure 3.2 HPLC elution profiles of extracts from roots of wild-type and mutant *Tagetes erecta*. The absorption was read at 340 nm. Compounds "7", "11", and "13" were identified as $AcOCH_2BBT$, BPT, and MeBBT respectively.

The chemical structures of all three unknown compounds were elucidated by recording GC/MS and NMR spectra of the purified compounds. Analysis of the spectra and comparison with known spectra from literature led to the unequivocal identification of compounds "11", "13" and "7" as BPT, MeBBT and $AcOCH_2BBT$, respectively. All three compounds are C_{13} thiophenes (Fig. 3.3), as opposed to the major thiophenes in wild-type *Tagetes erecta* which have a basic C_{12} carbon skeleton (Fig. 3.1). BPT is a monothiophene, whereas the other two compounds are bithienyls.

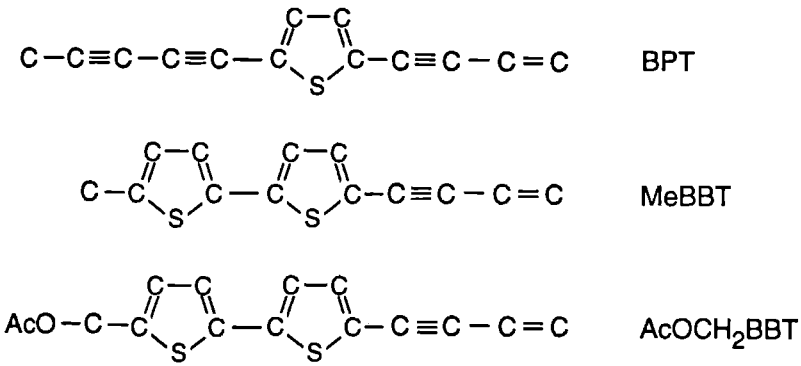

Figure 3.3 Molecular structures of the C_{13} thiophenes that accumulate in mutant *Tagetes erecta*.

An M3 progeny could be obtained only from one M2 plant in each of the two M2 sibling groups in which mutants originally segregated. Both M2 plants from which a progeny was obtained contained high amounts of BPT. The two M3 lines were designated line 70.122 and line 79.165.

Segregation analysis and inheritance

Segregation for BPT content was studied in two groups of M2 siblings raised from seeds of the two M1 flowers that originally segregated the mutants, and in the M3 progenies of the only two mutant M2 plants from which viable seeds were obtained (Fig. 3.4). The M2 data, assuming non-chimerism of the individual M1 flowers, suggest that high BPT content is a recessive trait because the high-BPT plants constitute a minority of the two M2 lines. This conclusion is confirmed by the presence of exclusively high-BPT plants in the two M3 lines. However, since approximately half the M2 plants contain a low level of BPT, which is not found in the wild type, some expression of the

mutation in the heterozygotes seems to occur and the mutation should therefore be described as being incompletely recessive. MeBBT and elevated levels of $AcOCH_2BBT$ were observed in M2 plants always, and only when BPT was present.

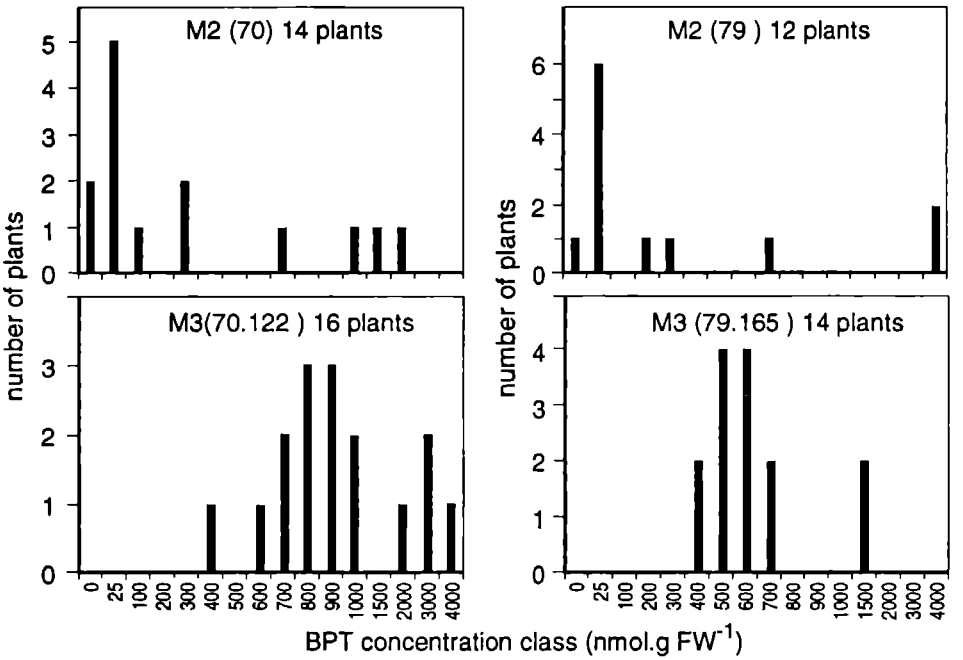

Figure 3.4 Segregation for BPT content in roots of two M2 sibling groups originating from two M1 flowers (nr. 70 and nr. 79), and in roots of two M3 lines (70.122 and 79.165) originating from one high-BPT plant in each of the M2 sibling groups. On the horizontal axis the upper limit of the BPT concentration of each class is indicated.

The concentrations of the various C_{12} and C_{13} thiophenes in M2 plants were related to eachother in a rather complex manner. Concentrations of BPT and $AcOCH_2BBT$ showed a weak positive correlation in M2 plants. M2 plants with very low or no BPT tended to have higher BBT and BBTOAc levels than

plants with higher BPT. On the other hand, above a "threshold" level of BPT, the concentrations of BBT and BBTOAc seemed to be positively correlated with the BPT concentration (data not shown). In roots of M3 plants on the other hand, as well as in transgenic hairy roots derived of M3 plants, the concentrations of all five thiophenes were strongly correlated (data not shown). The differences between the M2 and M3 generations were to be expected because M3 populations obtained by selfing are genetically more homogeneous than M2 populations.

The fact that all three C_{13} thiophenes always co-occurred indicated that probably one mutation caused accumulation of these compounds. All M4 and M5 plants analyzed so far (12 and 3 individuals, respectively) contained high concentrations of BPT, MeBBT and $AcOCH_2BBT$ which supports the conclusion of an inherited new trait. Together, the observations in both mutant lines indicated that one incompletely recessive mutation caused accumulation of all three C_{13} thiophenes, while at the same time inducing only a slight reduction of the synthesis of C_{12} thiophenes.

Characterization of M3 plants

Having established that M3 plants of both lines were homozygous with respect to one mutation of thiophene metabolism, attention was fucussed on analysis of the effects of the mutation on the distribution of the various thiophenes in organs of M3 plants.

As in wild-type plants, roots and hypocotyls were found to be the major organs for thiophene accumulation in both M3 lines (Table 3.1). Thiophenes were only incidentally detected at low concentrations in leaves of control and M3 plants. BPT was by far the most abundant thiophene in roots and hypocotyls of the mutant plants at the age of three weeks. Also MeBBT and $AcOCH_2BBT$ were prominent in these organs of mutant plants. In contrast, $AcOCH_2BBT$ was the only C_{13} thiophene found at low concentrations in roots

and hypocotyls of wild-type plants. BBT and BBTOAc were present in roots and hypocotyls of mutants at lower or similar concentrations than in the wild type. The concentrations of all five thiophenes were higher in M3 plants of the 79.165-line than in M3 plants of the 70.122-line.

Closer examination of M3 plants showed that, at the age of three weeks, plants of both M3 lines were reduced in size (55% to 77% of wild type size) and biomass (22% to 29% of wild type fresh weight) compared to control plants. After continued growth in the greenhouse in soil, the mutant plants significantly caught up by weight but remained shorter than wild-type plants (Fig. 3.5). The leaves of 70.122-type M3 plants were lighter green than those of wild type plants, whereas the leaves of 79.165-type M3 plants had a more rounded shape and a darker green color. The deviations of morphology and growth rate of mutant plants could be the result of background mutations or of pleiotropic effects of the mutation that caused accumulation of C_{13} thiophenes.

Table 3.1 Thiophene concentrations in roots and hypocotyls of plants of two M3 lines and control plants at the age of three weeks.
Concentrations (nmol.g FW^{-1}) are the means of determinations on eight plants ±se.

thiophene	hypocotyls			roots		
	wild type	M3(70.122)	M3(79.165)	wild type	M3(70.122)	M3(79.165)
BBT	0 0 ± 0 0	5 4 ± 3 6	6 4 ± 3 8	71 5 ± 12 6	17 5 ± 3 9	28 8 ± 3 9
BBTOAc	137 4 ± 12 0	59 2 ± 11 2	89 2 ± 10 2	57 5 ± 4 0	48 2 ± 5 9	63 6 ± 7 5
BPT	0 0 ± 0 0	140 5 ± 26 4	363 3 ± 79 4	0 0 ± 0 0	287 4 ± 35 3	419 3 ± 46 1
$AcOCH_2BBT$	0 9 ± 0 2	46 1 ± 10 6	90 4 ± 13 2	0 1 ± 0 1	43 8 ± 6 0	61 4 ± 5 6
MeBBT	0 0 ± 0 0	0 0 ± 0 0	26 6 ± 14 6	0 0 ± 0 0	61 6 ± 12 7	111 0 ± 18 8
total	138 3 ± 12 0	251 2 ± 19 7	575 9 ± 54 6	129 1 ± 9 2	458 5 ± 25 7	684 1 ± 30 6

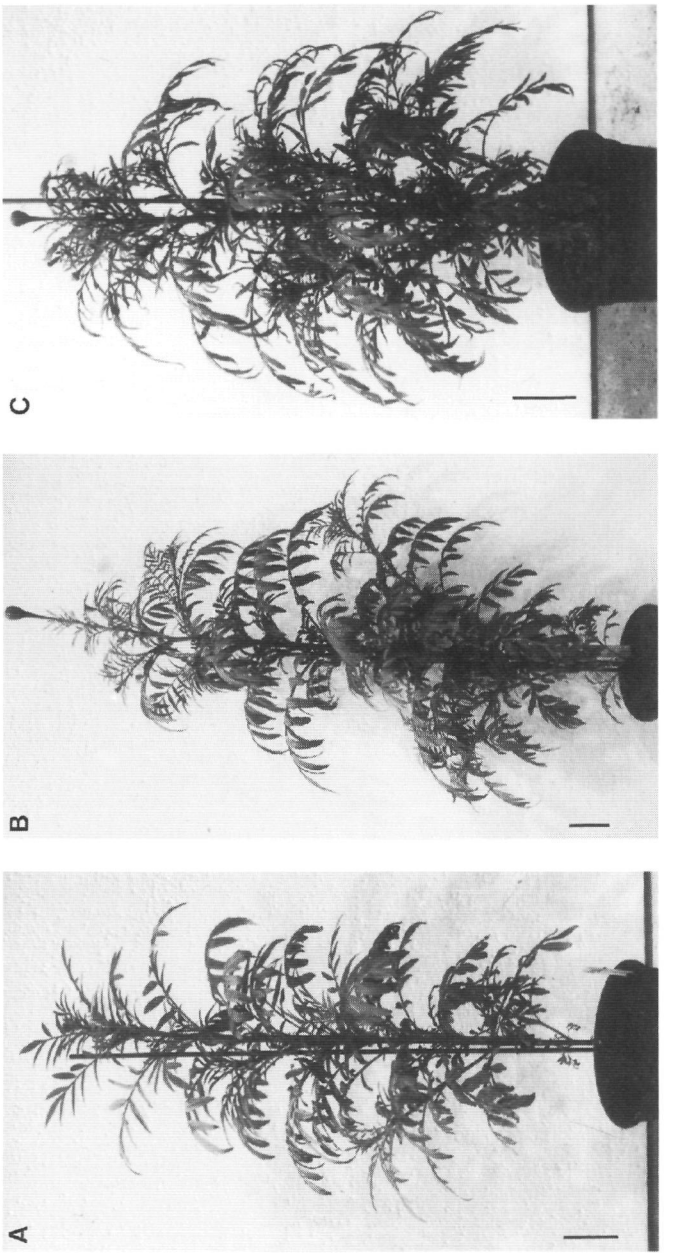

Figure 3.5 Comparison of wild-type *Tagetes erecta* (B) to mutant plants of M3 line 70.122 (A) and M3 line 79.165 (C) at the age of three months. Bars indicate 5 cm.

Mutant-derived root cultures

To obtain an unlimited supply of tissue for biochemical characterization of the mutant phenotype, hairy roots were produced which can be cultured indefinitely. Transgenic root cultures were obtained after transformation of M3 plants of both mutant lines with *Agrobacterium rhizogenes*. Transformation was ascertained by opine assays for 10 independent 70.122-type and 11 independent 79.165-type root clones (not shown). All mutant-derived root clones accumulated the C_{13} thiophenes as well as the C_{12} thiophenes whereas a wild type-derived root clone only produced C_{12} thiophenes. The mutant-derived roots greatly varied in morphology and growth rate. Total thiophene content of individual clones varied from 50 to 1,500 nmol per gram fresh weight, with BPT contributing more than half of the total thiophene content in all cases. As in the M3 plants, the concentrations of the major thiophenes were strongly correlated ($r > 0.90$ with $p < 0.02$), regardless of the morphology and growth rate of the individual root clones. This indicated that the ratios between the individual thiophenes are determined independent of the total thiophene content.

Metabolic characterization of mutants

A mutant (70.122-type) and a wild-type root clone that both grew well and produced amounts of common and mutant-specific thiophenes sufficient for accurate determination, were selected for experiments on the metabolic characterization of the mutant phenotype. Radioactive BPT and MeBBT were fed to mutant and control tissues and after incubations of 9, 21, or 44 hours the thiophenes were extracted and analyzed by HPLC. Radioactivity was counted in all fractions.

The monothiophene BPT was predominantly converted into BBT and BBTOAc by control roots (Fig. 3.6). A smaller portion of the BPT was converted into MeBBT and $AcOCH_2BBT$. The mutant roots converted BPT into MeBBT and $AcOCH_2BBT$ at about the same rate as the control roots, but only very small amounts of radioactive BBT and BBTOAc were produced (Fig. 3.6). Thus it seemed that the mutant is impaired in removal of the terminal methyl group from BPT.

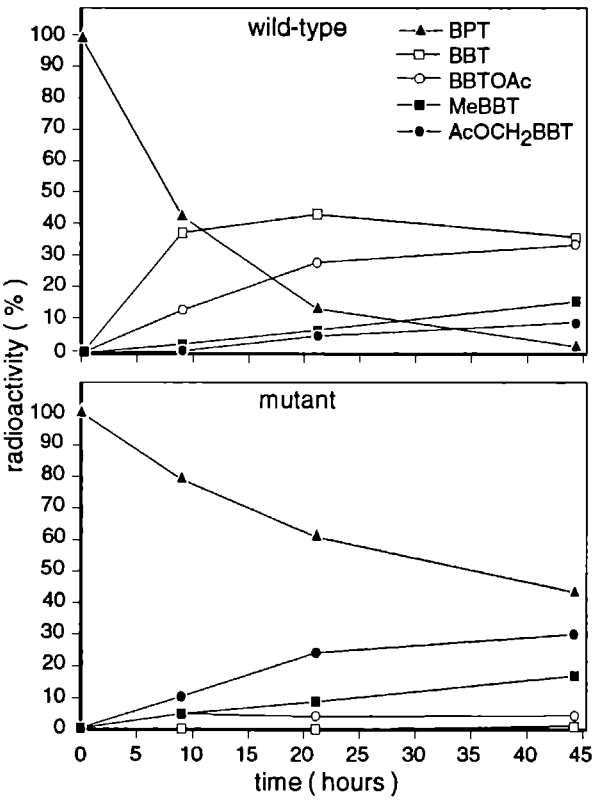

Figure 3.6 Conversion of ^{35}S-labeled BPT into C_{12} and C_{13} bithienyls by hairy roots derived from wild-type and mutant (70.122) *Tagetes erecta*. Total radioactivity in thiophenes, which remained constant, was taken as 100 %. Data are the means of determinations from two feeding experiments.

MeBBT was not converted to BBT or BBTOAc by either the mutant or the control roots. Instead, $AcOCH_2BBT$ was by far the major product after an incubation for 44 hours, both in wild type and mutant roots (Table 3.2). This indicated that MeBBT is a poor substrate for demethylation both in wild-type and mutant tissues.

When BPT and MeBBT were fed to whole plants of mutant and wild-type *T. erecta*, the same bioconversions were observed in the attached roots as in the root cultures. No BPT was converted by tomato roots, which indicates that the bioconversions required conditions specific to *Tagetes*.

Table 3.2 Distribution of radioactivity (%) after feeding for 44 hours of ^{35}S-labeled MeBBT to hairy roots derived from wild-type and mutant (70.122) *Tagetes erecta*.
Total radioactivity in thiophenes, which remained constant, was taken as 100 %. Data are the means of determinations from two feeding experiments ±se.

compound	wild-type	mutant
MeBBT	62.6 ± 11.4	19.2 ± 1.7
AcOMeBBT	34.2 ± 8.1	72.4 ± 0.8
unidentified	3.3 ± 3.3	8.5 ± 0.9
BBT, BBTOAc	0	0

DISCUSSION

Mutants of thiophene metabolism were generated by treating seeds of *Tagetes erecta* with EMS and identified by HPLC analysis of root extracts from M2 plants. Two mutants were identified which may, however, have originated from the same mutational event. Large amounts of one C_{13} monothiophene and two C_{13} bithiophenes, which are absent or appear at low concentrations in the wild

type, accumulate in roots and hypocotyls of mutant plants. Precursor-feeding experiments with plants and a root culture of one mutant line indicate that the mutation does not affect the plant's ability to form the second thiophene ring, but rather its ability to remove the terminal methyl group from the mono-thiophene.

Mutants of thiophene metabolism were identified after screening an M2 population of 338 plants. In *Arabidopsis*, a desired selectable loss-of-function mutant can usually be found among 2,000 heavily mutagenized M2 plants (Haughn & Somerville, 1987). The retrieval, in this investigation, of mutants from a much smaller population may be due to the fact that each one of a range of mutations could have yielded a phenotype that would have been detected in the applied screening protocol. These results indicate that the mutagenesis approach is very effective for detecting metabolic mutants, especially in *Tagetes erecta*.

Genetic analysis of the mutants was hampered by the fact that M3 progenies were obtained only from two M2 plants, which both appeared to be homozygous for the mutation, since all M3 plants contained high BPT levels. If our assumption of a monogenic, incompletely recessive trait is correct, the progeny of a heterozygous M2 plant should again have segregated in a 1:2:1 ratio. Another handicap in the genetic analysis of the mutants is the impracticability of emasculation of marigolds for the purpose of backcrossing. Due to these limitations for genetic analysis, it is at this stage impossible to distinguish pleiotropic effects caused by a mutation of thiophene metabolism from the effects of background mutations. Also, it is impossible to decide whether the two mutants of thiophene metabolism are independent. As a result of the method that was applied for mutagenesis, background mutations would be expected (Haughn & Somerville, 1987) which may be responsible for the deviant leaf shape or color and the slower growth of the mutants. Since the thiophene profiles of both mutants are highly comparable, it would be reasonable to assume that the M3 lines have arisen by segregation from one original, heterozygous, mutant. However, given the morphological differences

between the M3 lines, it cannot be excluded that both lines stem from independent mutational events.

High levels of four C_{12} thiophenes (BBT, BBTOAc, BBTOH, α-T) have previously been found in *T. erecta* and the related *T. patula* (Sütfeld, 1982; Mukundan & Hjortso, 1990; Jacobs *et al.*, in press; Chapter 2 of this thesis). Low concentrations of some C_{13} thiophenes have been incidentally reported as well (Bohlmann *et al.*, 1967; Groneman *et al.*, 1984; Ketel, 1986; Caniato *et al.*, 1990). In the previous chapter of this thesis, it was shown that $AcOCH_2BBT$ and BPT occur at low concentrations in *T. patula* (Jacobs *et al.*, in press), and in this chapter it is shown that $AcOCH_2BBT$ is present at low concentrations in roots and hypocotyls of wild-type *T. erecta*. However, the presence of three C_{13} thiophenes, BPT, MeBBT and $AcOCH_2BBT$, at high concentrations, is clearly a new trait of the mutant plants.

The distribution pattern of thiophenes over different plant organs was identical in mutant and wild-type plants, and the mutant phenotype was preserved in hairy root cultures. These findings indicate that regulatory mechanisms that cause different accumulation levels in the individual organs have not been affected by the mutation. The total concentration of thiophenes in roots and hypocotyls of mutant plants was significantly higher than in wild-type plants. This may be related to the slower growth rate of mutant plants compared to wild-type plants, which was probably caused by background mutations. Another explanation could be that C_{12} bithienyls are more effective feed back inhibitors of their own synthesis than the C_{13} thiophenes.

The C_{13} thiophenes fit the accepted biogenetic scheme for thiophene synthesis that is largely based on structural relationships. However, the results of kinetic studies presented here make an important change in this scheme necessary. Firstly, it was shown that C_{12} bithienyls (BBT, BBTOAc) and C_{13} bithienyls (MeBBT, $AcOCH_2BBT$) are formed from BPT in mutant and wild-type *Tagetes erecta*. Although expected, this had not been previously demonstrated. The feeding experiments clearly showed that MeBBT is not an intermediate in the formation of BBT, as had previously been claimed (Bohlmann & Hinz, 1965;

fig. 1.2). Instead, it seems that BPT represents a branching point, with one branch leading to MeBBT and its derivatives and the other branch leading to BBT and its derivatives. Apparently, removal of the terminal methyl group is more difficult or impossible once closure of the second ring has been completed. Thus, in the formation of BBT, demethylation of BPT must precede formation of the second thiophene ring (fig. 1.2). Removal of the terminal methyl group prior to second ring formation supposes the existence of a C_{12} monothiophene. Such a compound is indeed present in the related species *Eclipta erecta* (Bohlmann & Zdero, 1970).

Secondly, the feeding experiments showed that the mutation, at least in one mutant line, affects the efficiency of removal of the terminal methyl group. Control hairy roots convert BPT mainly into C_{12} bithienyls, which involves demethylation, whereas in the mutant hairy roots the C_{12} bithienyls are only minor products. Thus, it seems that the mutant is deficient in demethylation of C_{13} thiophenes. Demethylation of BPT is probably catalyzed by a cytochrome P-450 enzyme. These enzymes, which occur widely both in eukaryotes and prokaryotes, catalyze a variety of oxidation reactions in which molecular oxygen is used as a substrate. In eukaryotes, the enzymes are membrane-bound and often involved in detoxification or in metabolism of relatively non-polar substrates (Ortiz de Montellano, 1986). Relatively little is known of these enzymes in plants, but functions in biosynthesis of fatty acids, hormones, and secondary metabolites, and in detoxification of xenobiotics have been established (O'Keefe *et al.*, 1987; Donaldson and Luster, 1991; Durst, 1991). Only a few plant cytochrome P-450 enzymes have been cloned (Bozack *et al.* 1990; Meijer *et al.* 1993). In a mutant strain of *Saccharomyces cerevisiae*, inactivation of a cytochrome P-450 involved in demethylation of lanosterol was found to be due to substitution of a single amino acid (Ishida *et al.* 1988). Since EMS treatment causes mainly point mutations (Haughn and Somerville 1987) it is possible that also a putative BPT-demethylase was inactivated by an amino acid substitution resulting from a point mutation.

Thirdly, the feeding experiments suggest that closure of the second

thiophene ring proceeds most efficiently if it occurs in concert with, or after removal of the terminal methyl group. This is concluded from the faster formation of C_{12} bithienyls compared to the slower formation of C_{13} bithienyls by wild-type roots. If demethylation would facilitate formation of the second ring this would be in line with results of feeding experiments on the formation of the third thiophene ring (Jente *et al.*, 1981). They found that two C_{12} bithienyldiynes were converted 30 to 100 times more efficiently into *α*-T than the corresponding methyl-bithienyldiynes were converted into Me-*α*-T.

Species of the genus *Echinops*, which is related to *Tagetes*, can be categorized into two groups: species in one group contain various C_{13} mono- and bithienyls and C_{12} bi- and terthienyls that are all derived from BPT, whereas species in the other group contain C_{13} bithienyls and C_{12} bi- and terthienyls but not the C_{13} monothienyls. The *Tagetes* mutants that we created would fit into the former group, whereas wild-type *T. erecta* would better fit in the latter. One may speculate that the *Echinops* species which do contain C_{13} monothiophenes are, like the *Tagetes* mutant, less efficient in removal of the terminal methyl group compared to the species in the other group. Thus the separation into two groups, may originate from a natural mutation in *Echinops*.

As in wild-type plants, no pentaynene nor any other polyacetylene was detected in the mutants. This is not surprising, because thiophene ring formation was not affected in the mutants and total thiophene concentration in the mutants was even higher than in wild-type plants.

The relative ease with which mutants of thiophene synthesis could be found should be an encouragement for further screening. The enzymes involved in formation of polyacetylenic triple bonds and in thiophene ring formation would be attractive targets for mutagenesis, since these enzymes are unique and uncharacterized. Mutants affected in these enzymes might be recognized by the absence/appearance of certain thiophenes and/or by the accumulation of various poly-acetylenes. The mutants that have been described here will be valuable instruments in work aimed at characterization of the demethylating enzyme, which represents a class of poorly characterized enzymes in plants.

CHAPTER 4

THIOPHENE BIOCONVERSIONS IN *TAGETES* PROTOPLASTS

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ABSTRACT

The thiophene content of *Tagetes* protoplasts isolated from various organs, and the ability of these protoplasts to convert or synthesize thiophenes were investigated and compared to the thiophene content and metabolic capacities of intact organs. It was found that a substantial fraction of thiophenes is localized intracellularly in hypocotyls. Another significant fraction appears to be localized in the extracellular space. Protoplasts originating from hypocotyls and leaf blades are equally efficient in formation of the second thiophene ring and in hydroxylation and acetylation of thiophenes although the organs from which these protoplasts are isolated differ greatly in thiophene content and thiophene synthesizing capacity. All reactions of thiophene synthesis from formation of the first ring onwards can be carried out by hypocotyl protoplasts.

INTRODUCTION

Secondary metabolism is a general phenomenon in plants. Within plants, secondary compounds usually accumulate only in specific organs or tissues and are lacking or present at very low concentrations in others. In a number of cases, it was demonstrated that accumulation of secondary compounds is closely related to the expression of structural genes of the corresponding secondary pathway (Meijer *et al.*, 1993; Koes *et al.*, 1994). An organ that appears to be inactive in a particular pathway may nevertheless possess the capacity to carry out reactions of that pathway. Such bioconversion capabilities may remain unnoticed because precursors are not transported into the organ or because the products are rapidly degraded or exported out of the organ.

Protoplasts are generally considered a valid cellular model for physiological and biochemical investigations (Webb and Williams, 1984; Percival *et al.*, 1991; Pomeroy and Mudd, 1993) and a valuable tool for compartmentation studies (Sitbon *et al.*, 1993). After liberation from plant

tissues, protoplasts largely retain the responsiveness to hormonal and environmental signals of the original tissues (Dangl *et al.*, 1987; Huttly and Baulcombe, 1989; Bach *et al.*, 1993). In investigations of secondary metabolism, protoplasts offer a number of potential advantages over intact plants. Firstly, they may be used to establish or disprove the intracellular localization of secondary compounds and the enzymes and substrates involved in their formation. Secondly, protoplasts may be used to determine whether cells are able of certain metabolic activities, independent of the structures and compounds by which they are surrounded *in planta*, and if not, which of these structures or compounds need to be supplied for restoration of activity. Thirdly, the conditions for certain bioconversions can be easily optimized in a protoplast system and transport-related problems are avoided, thereby creating the possibility of revealing biosynthetic capacities in the isolated cells that remain unnoticed in the intact organs. Furthermore, protoplasts constitute a "semi" *in vitro* system which may be exploited for the development of a true *in vitro* assay for a specific bioconversion.

Tagetes species (marigolds) produce thiophenes, nematicidal, sulfur-containing secondary compounds (Uhlenbroek and Bijloo, 1958; 1959). The major thiophenes of *Tagetes*, BBT and BBTOAc, accumulate mainly in the roots and hypocotyls whereas the concentrations in leaves are low (Sütfeld, 1982; Downum and Towers, 1983; Jacobs *et al.*, in press). The bithiophenes of *Tagetes* are synthesized from an oleic acid-derived polyacetylenic precursor, tridecapentaynene (PYE) (Bohlmann and Hinz, 1965; Bohlmann *et al.*, 1966; Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1985). The sequential formation of two adjacent sulfur-containing aromatic rings at defined positions in the polyacetylene substrate is the most characteristic feature of thiophene synthesis. In addition, removal of a terminal methyl-group is required to obtain the C_{12} thiophenes that predominate in *Tagetes*. Acylation and hydroxylation of the side chain lead to the more polar thiophenes. Until now, no enzymes of this metabolic pathway have been characterized or isolated.

In chapter 2 of this thesis, it was shown by feeding [^{35}S]sulfate to plants that thiophene synthesis takes place in roots and hypocotyls of *Tagetes* plants, whereas in leaves the biosynthetic activity is very low (also: Jacobs *et al.*, in press). As illustrated in chapter 3, various modifications of thiophenes (hydroxylation, acetylation) can be conveniently studied in (cultured) *Tagetes* roots. Until now, the capacity of leaves to modify thiophenes could not be adequately investigated since thiophenes are poorly transported in intact plants (Jacobs *et al.*, in press; Chapter 2 of this thesis).

Radiolabeled thiophenes purified from young plants can be used to evaluate the bioconversion capabilities of protoplasts originating from various organs. The C_{13} monothiophene BPT, which accumulates in a mutant of *T. erecta* (Chapter 3 of this thesis), may be used to study the formation of the second thiophene ring and the removal of the terminal methyl group. The C_{13} bithiophene MeBBT from the same mutant is a suitable precursor to study demethylation, hydroxylation and acetylation. The C_{12} thiophene BBT, which is the major thiophene in wild-type *Tagetes* may also be used to study the latter two modifications.

In this chapter, thiophene concentrations of intact *Tagetes* protoplasts are reported and compared to the content of intact organs. Furthermore, results are presented of thiophene feeding experiments with isolated *Tagetes* protoplasts and, for comparison, organs of *Tagetes* plants. Three ^{35}S-labeled thiophenes, BBT, MeBBT and BPT, were used as substrates to study the bioconversion capacities of hypocotyl- and leaf-derived protoplasts in relation to the biosynthetic capacities of the intact organs. In addition, the capacity of hypocotyl-derived protoplasts to synthesize thiophenes *de novo* was investigated by feeding ^{35}S-labeled cysteine. The experiments represent a first step towards the development of *in vitro* assays for specific thiophene bioconversions.

CHAPTER 4

MATERIAL AND METHODS

Plant material

Plants of *Tagetes erecta* L. and *T. patula* L cv. "Nana" were grown during the summer under natural light conditions in a greenhouse in soil until the age of three weeks. Plants of *T erecta* were also grown aseptically in B5 medium that was solidified with 0.2 % (w/v) Gelrite. B5 medium contained Gamborg's nutrient solution (Gamborg, 1970), sucrose (85 mM), and biotin (100 $\mu g.l^{-1}$). *In vitro* grown plants were kept in a growth chamber at 25 °C and 70% relative humidity with a 16-hour photoperiod, until the age of three weeks.

Protoplast preparation

Protoplasts were prepared from hypocotyls of three-weeks-old soil-grown plants and from leaf blades of three-weeks-old *in vitro* grown plants About 100 hypocotyls were longitudinally cut once or about 1 g of freshly harvested leaf blades were cut into pieces of 1 to 4 mm^2. After cutting, the tissues were incubated in a Petri dish containing 10 ml protoplast digestion mix, on a slowly rotating platform for 4 to 5 hours at 28 °C in the dark. The digestion mix consisted of 5% (w/v) Cellulase "Onozuka R-10" and 0.5% (w/v) Macerozyme R-10 (both from Serva) in AS-medium (sucrose, 0.4 M, KH_2PO_4, 200 μM; KNO_3, 1 mM; $MgSO_4$, 1 mM, KI, 1 μM, $CuSO_4$, 100 nM; $CaCl_2$, 10 mM; MES, 500 μM; pH 5.8). The digested tissues were sieved through a household sieve and washed with two volumes of AS-medium. The protoplast suspension was transferred to four 10-ml test tubes and centrifuged for 5 minutes at 75 x g. The floating protoplasts were collected using a wide-bore pipette and diluted in five volumes of AS-medium. Centrifuging, diluting and collecting of protoplasts were repeated two more times Protoplast numbers, thiophene and protein concentrations were determined in the final layer of floating protoplasts and in a sample of AS-medium from the middle of the test tube. The diameter of at least 100 protoplasts from each source was measured under a microscope in a haemocytometer.

Protein determination

Protein concentrations were determined using the Bradford assay (Bradford, 1976). Tissue samples were thoroughly ground in 1 ml PBS buffer (NaCl, 0.138 mM, KCl, 2.7 mM, Na_2HPO_4,

10 mM; KH_2PO_4, 1.8 mM) and subsequently sonicated. Protoplasts were sonicated in 1 ml AS-medium. After sonication, the samples were centrifuged for 5 minutes at 15,000 x g and the supernatant was used for the assay in an appropriate dilution.

Thiophene analysis

Thiophenes were extracted and analyzed as described (Croes *et al.*, 1989[a]). Protoplasts and tissue samples were ground in ethanol: water (1:1 v/v) and thiophenes were partitioned into a mixture of hexane: *t*-butylmethylether (1:1 v/v). The organic solvents were evaporated under nitrogen gas and the thiophenes were recovered in a small volume of ethanol. The extracts were subjected to HPLC analysis on a Lichrosorb RP-18 column using acetonitrile:water (70 30) as an eluent at an elution rate of 1.5 ml.min^{-1}. The thiophenes in the eluate were quantified on basis of their UV absorption at 340 nm. The eluate of radioactive extracts was collected in 75 fractions of 0.5 ml per run and radioactivity in all fractions was measured using a liquid scintillation counter.

Preparation of radioactive thiophenes

Radioactive thiophenes were obtained by incubating young plants for several days in 10 ml growth medium containing 15 MBq $^{35}SO_4^{2-}$ per plant (specific activity 750 MBq.mmol^{-1}). Thiophenes were extracted as described and purified by subjecting highly concentrated extracts to preparative HPLC on a Lichrosorb RP-18 column with acetonitrile:water (65:35) as the eluent. Selected fractions were pooled, the eluent was evaporated under nitrogen gas and the purified thiophenes were dissolved in ethanol. Purity of the isolated compounds was confirmed by analytical HPLC.

Feeding experiments

Intact shoots and detached leaves (petiole plus leaf blade) were incubated for 16 hours at 25 °C in the dark in vials containing 500 µl half-strength Hoagland solution to which ^{35}S-labeled thiophene was added. Batches of $3x10^6$ protoplasts were incubated with ^{35}S-labeled substrates in 3 ml AS·B5 medium (1:1), supplemented with NAA (50 ng.l^{-1}) and BAP (100 ng l^{-1}), for 16 hours at 25 °C in the dark, in 50-ml flasks mounted on a wheel rotating at 2 rpm. The following amounts of ^{35}S-labeled thiophenes in 10 µl ethanol were added per experiment: 22.5

nmol BBT at a specific activity of 90 Bq.nmol^{-1}; 7 nmol MeBBT at 400 Bq.nmol^{-1}; 18 nmol BPT at 150 Bq.nmol^{-1}. In some experiments, 0.31 pmol ^{35}S-labeled cysteine at 48 TBq.mmol^{-1} (Amersham) in 10 μl water was fed to hypocotyl protoplasts. At the end of the incubations, thiophenes were immediately extracted from intact plant parts and stored at -20 °C until analysis. Protoplast morphology was investigated under the microscope. Subsequently, one volume of 96% ethanol was added and the protoplasts were stored at -20 °C, or thiophenes were extracted immediately.

RESULTS

Thiophene contents of hypocotyl-derived protoplasts

To investigate whether thiophenes are localized intracellularly, thiophene concentrations were determined in hypocotyl-derived protoplasts of young *Tagetes* plants and compared to the content of intact hypocotyls. Protoplasts from hypocotyls of both *Tagetes* species did contain considerable amounts of thiophenes (Table 4.1). The concentrations in protoplasts of both species were comparable when expressed as nmols per g fresh weight or as nmols per mg protein, as was also true for intact hypocotyls. On a per protoplast basis, *T. patula* protoplasts contained much larger amounts of thiophene than those of *T. erecta*, which was entirely due to the three times larger volume of *T. patula* protoplasts. The comparison of thiophene concentrations in protoplasts and intact hypocotyls (nmol.g FW^{-1}) indicated that probably a large fraction of the thiophenes is localized extracellularly.

Table 4.1 Thiophene concentrations in intact hypocotyls and in protoplasts derived from them. Data are averages of four determinations.

Tissue	Thiophene concentration expressed as	*T. patula*	*T. erecta*
Intact	nmol.(g FW)$^{-1}$	93.6	109.8
	nmol.(mg protein)$^{-1}$	39.5	58.1
Protoplasts	nmol.(g FW)$^{-1}$	71.3 [1)]	61.3 [1)]
	nmol.(mg protein)$^{-1}$	14.4	11.4
	nmol.(10^6 protoplasts)$^{-1}$	6.3	2.9

[1)] Calculated by using the average volume of a protoplast which was determined by measuring the diameter of 100 protoplasts, and assuming that 1 μl equals 1 mg.

Thiophene bioconversions in protoplasts

Thiophene bioconversions were studied in protoplasts of *T. erecta* and, for comparison, in intact organs. Protoplasts from hypocotyls and leaf blades, as well as whole shoots and detached leaves were incubated with three ^{35}S-labeled thiophenes. After 16 hours, thiophenes were extracted from the protoplasts, the petioles and leaf blades of the detached leaves, and the hypocotyls of the shoots. Distribution of radioactivity over non-polar, sulfur-containing compounds was determined by HPLC separation and scintillation counting of fractions that were collected from the HPLC column.

Approximately 80% of the radioactivity that was added as ^{35}S-labeled thiophenes was retrieved in discrete peaks, indicating that no significant degradation of thiophenes to polar sulfur-containing compounds took place, neither in protoplasts, nor in intact tissues.

In intact hypocotyls and petioles, BBT and MeBBT were mainly converted to their respective hydroxy- and acetoxy-derivatives, whereas BPT was

converted to BBT, BBTOAc, $AcOCH_2BBT$, BBTOH and $HOCH_2BBT$ (Table 4.2). All three thiophenes were taken up very poorly into the leaf blades and thus no bioconversions could be observed in intact leaf blades.

In protoplasts derived from hypocotyls and leaf blades, BBT and MeBBT were converted to their hydroxy- and acetoxy-derivatives (Table 4.3). However, compared to intact tissues, the ratio between both types of products was shifted towards the hydroxy-derivatives. Additional relatively polar unidentified products were detected upon feeding of BBT and MeBBT to leaf blade protoplasts and, to a lower extent, upon feeding to hypocotyl protoplasts (Table 4.3). These products were not formed in the intact organs (Table 4.2). BPT was converted by the protoplasts from hypocotyls and leaf blades to BBTOAc and $HOCH_2BBT$ and to several unidentified polar compounds (Table 4.3). No bioconversions occurred in sonicated protoplasts with any of the substrates.

Table 4.2 Conversion of ^{35}S-labeled thiophenes by intact tissues of *T. erecta*

substrate	tissue	% of radioactivity in products						
		BBTOH	BBTOAc	BBT	$HOCH_2BBT$	$AcOCH_2BBT$	MeBBT	BPT
BBT	hypocotyl	9.9	30.2	59.9				
	petiole	9 8	33.4	56 8				
MeBBT	hypocotyl			0.7	3 1	29.7	65.0	1.4
	petiole			0 7	6 0	34.2	55 4	3.7
BPT	hypocotyl	3.1	19.2	7.3		1.4		69 1
	petiole		4.7	2 6	4.1	2 1		86.5

Shoots and detached leaves were incubated for 16 hours in medium containing one of three radioactive thiophenes. Radioactivity in thiophenes was determined in the hypocotyls of the shoots and in the petioles of the detached leaves. Total radioactivity in thiophenes was set at 100 %. Data are the means of two experiments.

Table 4 3 Conversion of ^{35}S labeled thiophenes by isolated protoplasts of *T erecta*

substrate	protoplast source	% of radioactivity in products										
		a	b	c	d	OH	OAc	BBT	$HOCH_2$	$AcOCH_2$	MeBBT	BPT
BBT	hypocotyl		0 9			10 1	3 4	85 6				
	leaf blade		3 0			3 3	2 5	91 2				
	leaf blade	2 5	18 5			15 6	1 3	62 2				
MeBBT	hypocotyl								1 7	2 9	95 4	
	leaf blade			7 1					5 7	3 7	83 6	
	leaf blade			2 7			0 1		0 9	0 1	96 2	
BPT	hypocotyl		0 5				0 5		0 1			98 9
	hypocotyl		4 0				0 5		0 6		0 1	94 8
	leaf blade		8 8	43 5			3 5		6 4			37 8
	leaf blade		2 2		0 2		2 2					95 4

Conversions in protoplasts were performed with 10^6 protoplasts per experiment Results of individual experiments are presented Compounds designated a, b, c, d elute early from the HPLC column and are unidentified, BBTOH, BBTOAc, $HOCH_2BBT$, and $AcOCH_2BBT$ are abbreviated to OH, OAc, $HOCH_2$, and $AcOCH_2$ respectively

Thiophene synthesis in protoplasts

To determine if all characteristic steps of thiophene synthesis occurred in isolated wall-less cells, hypocotyl protoplasts of *T. erecta* were incubated with ^{35}S-labeled cysteine. A low percentage (on the average 0.3 %) of the added radioactivity was indeed incorporated into several thiophenes, mostly BBT (Table 4.4). The percentage incorporated varied with each experiment and was rather unpredictable. Addition of ATP (5 μmol ml^{-1}), NADPH (100 nmol ml^{-1}) or pyridoxal-5-phosphate (25 nmol ml^{-1}) did not improve the yield of radioactive

thiophenes, nor did addition of PYE, the presumed precursor of thiophenes in *Tagetes*. No incorporation at all occurred in sonicated protoplasts.

Table 4.4 Incorporation of ^{35}S into thiophenes upon feeding of ^{35}S-cysteine to hypocotyl-derived protoplasts of *T. erecta*

compound	% of radioactivity in each compound
BBTOAc	14.0 ± 3.6
$AcOCH_2BBT$	8.1 ± 3.3
BBT	53.9 ± 10.5
BPT	12.7 ± 5.0

Approximately $3x10^6$ protoplasts were incubated for 16 hours in 3 ml medium containing 0.31 pmol ^{35}S-cysteine (48 $kBq.pmol^{-1}$). An average of 0.3 % of radioactivity was incorporated into thiophenes in each experiment. Data are means of 6 independent experiments ± se. The total radioactivity in thiophenes was set at 100 %.

DISCUSSION

The data presented in this chapter show that a significant fraction of thiophenes is stored intracellularly in hypocotyls of two *Tagetes* species. It is also strongly indicated that another significant part of the thiophenes is stored in the apoplast. Furthermore, it is demonstrated that the highly characteristic reaction of second thiophene ring formation and several important side chain modifications (hydroxylation, acetylation) are performed with equal efficiency by protoplasts originating from hypocotyls and leaf blades, two organs that greatly differ in thiophene content. Thus there seems to be no relationship between thiophene content and the capacity to perform these bioconversions. Feeding experiments with labeled cysteine showed that hypocotyl protoplasts are able to perform all reactions of thiophene synthesis from formation of the first

thiophene ring onwards. None of the conversions occurred in sonicated protoplasts, which indicates that an intact biological system is required for the reactions to take place and excludes the possibility that the observed conversions were uncatalyzed chemical transformations.

Conclusions with respect to thiophene localization in intact tissues should be drawn cautiously from data obtained for protoplasts. First of all, the various cell types, and the cells of various tissues (which can greatly differ with respect to thiophene content; see chapter 2) are probably not equally well represented in the total protoplast population prepared from an organ. Furthermore, the volume of an isolated protoplast is probably not equal to the volume of the original cell in the intact tissue. Therefore, the thiophene concentration in protoplasts on a per g fresh weight basis can be only an approximation of the actual intracellular thiophene concentration in the intact tissue, since the former was calculated using a protoplast fresh weight that was estimated on basis of the measured protoplast volume. Nevertheless, the data presented (Table 4.1) may be used to roughly calculate the distribution of thiophenes over apoplast and symplast. For example, if it is assumed that 95 % of all protein (by weight) is in the symplast, which is a reasonable estimate for hypocotyls of young plants, it follows that 19% (*T. erecta*) to 35% (*T. patula*) of the thiophenes are localized in the symplast. Assuming that the symplast constitutes 70% of a hypocotyl's fresh weight leads to a calculated fraction of symplastic thiophenes of 39% for *T. erecta* and 53% for *T. patula*. Interestingly, both calculations indicate that a larger fraction of thiophenes is localized intracellularly in *T. patula* than in *T. erecta*. These thiophenes do not leach out of the cells, since thiophene concentrations in protoplasts remained unchanged after dilution (not shown).

Several thiophene modifications (second ring formation, hydroxylation, acetylation) that had never been detected in intact leaves, were observed in leaf blade protoplasts. This suggests that in leaves thiophene molecules can be modified in several ways provided the necessary substrates are supplied directly to the cells.

In leaf blade protoplasts several unidentified compounds are formed to a much greater extent than in hypocotyl protoplasts and intact hypocotyls and petioles. If these compounds were degradation products of thiophenes, their occurrence would indicate that the low thiophene contents of leaves may result from thiophene degradation. However, it is more likely that these compounds are modified bithiophenes (*e.g.* BBTOHOAc, $BBT(OH)_2$, $BBT(OAc)_2$) which elute early from the HPLC column and which are known to occur in *Tagetes* or related species (Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1985). Identification of the unknown compounds was not yet possible due to the small differences in the retention times of these compounds. If the unidentified compounds indeed are polar bithiophenes, this would indicate that different thiophene modifications occur in leaf blade and hypocotyl protoplasts which might reflect similar differences between the intact organs. Alternatively, metabolic changes may have occurred as a result of protoplast isolation. Such metabolic alterations due to protoplast isolation have been observed in several other cases (Anderson *et al.*, 1982; Davies and Faciotti, 1984; Webb and Williams, 1984; Browse *et al.*, 1988; Pomeroy and Mudd, 1993).

It is believed that the protoplast system may be used advantageously to further investigate various aspects of specific conversions in thiophene synthesis. Optimization of the conditions for first and second thiophene ring formation will be helpful in the study of the mechanism and regulation of these very characteristic conversions and in the development of *in vitro* assays for enzyme purification.

CHAPTER 5

THE EFFECT OF SULFATE LIMITATION ON THIOPHENE BIOSYNTHESIS IN CULTURED *TAGETES* ROOTS

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ABSTRACT

The influence of the sulfate concentration in the growth medium on primary and secondary metabolism of cultured *Tagetes* roots was investigated. Under conditions of sulfate limitation, thiophene content and thiophene synthesis were significantly reduced at least five days before primary metabolism was affected. At the same time, the non-protein thiol content and the sulfate uptake capacity were not affected. Upon transfer of roots from sulfate-limited medium to medium containing normal amounts of sulfate, the thiophene synthesizing capacity began to recover after a lag phase of 8 hours and reached nearly normal levels after 24 hours. Recovery did not occur if an inhibitor of mRNA maturation was present. The data indicated that, in the case of sulfate limitation, the activity of thiophene synthesis is at least partially down-regulated at the level of gene expression. Precursor feeding experiments with radiolabeled thiophenes revealed that several steps in thiophene metabolism proceeded more slowly under sulfate-limiting conditions. The rates of conversions requiring a sulfur donor was more reduced than those of conversions not requiring a sulfur donor.

INTRODUCTION

Metabolic activity characterized as "primary metabolism" is essential for growth and development of organisms. A relatively small number of primary metabolic pathways are active in most cells of most organisms to supply the building blocks of cellular structures and the energy required for the organisms to proceed in their activities.

A large variety of specialized biochemical pathways occurring in a limited number of organisms are referred to as secondary metabolism. Within an organism, a specific secondary pathway usually is active only in specialized cells during a limited phase of development, or as a reaction to certain signals from

the environment (Wiermann, 1981; Luckner, 1990). Secondary compounds are usually not of immediate importance for survival of the producing cell, but rather are of general importance to the whole organism. A large variety of functions, reflecting the enormous structural diversity among secondary compounds have been proposed, mostly in communication and defense (Harborne, 1988).

The precursors of secondary metabolism are primary metabolites or nutrients which can also be used in primary reactions. Obviously, the synthesis of secondary compounds requires the input of energy. Consequently, a competition exists for substrates and metabolic energy between primary and secondary pathways, which requires the existence of mechanisms that regulate the flow of substrates into primary and secondary routes. Such regulatory mechanisms are especially important when the secondary compounds constitute a large fraction of the organism's dry weight or when their synthesis requires the input of large amounts of energy.

Since the availability of sufficient amounts of primary metabolites is essential for survival of an organism whereas it may function well in the absence of its typical secondary compounds, it may be predicted that the flow of a precursor into secondary pathways is selectively reduced if it becomes limiting. Accordingly, in the case of precursor shortage one would expect secondary metabolism to be down-regulated to a lower activity before primary metabolism is noticeably affected. This down-regulation may be achieved by kinetic or allosteric mechanisms involving differential affinity of competing enzymes for the limiting substrate. However, it is likely that also active regulatory mechanisms are involved which lead to inactivation or reduced availability of specific enzymes of secondary metabolism.

Cultured roots of *Tagetes patula* and *T. erecta* obtained through transformation by *Agrobacterium rhizogenes* LBA9402 (pRi 1855; Birot *et al.*, 1987) which grow rapidly on a simple hormone-free medium (Croes *et al.*, 1989[b], Mukundan and Hjortso, 1990) constitute an appropriate and convenient model to test this hypothesis. The roots actively synthesize thiophenes, secondary metabolites with a nematicidal activity (Uhlenbroek and Bijloo, 1958;

1959). Thiophene molecules consist of up to three heterocyclic aromatic rings, each containing one sulfur atom. The rings are formed by repeated addition of reduced sulfur from an unidentified thiol-donor to conjugated acetylenic groups of a precursor polyacetylene (Bohlmann and Hinz, 1965; Bohlmann *et al.*, 1966; Bohlmann *et al.*, 1973; Bohlmann and Zdero, 1985). The sulfur atoms of all thiophene rings ultimately come from the sulfate provided in the medium, which is thus used as a precursor for both primary and secondary metabolism. The rate of thiophene synthesis in *Tagetes* roots grown under various conditions can be accurately quantitated (Croes *et al.*, 1993) and the availability of sulfate can be easily modified.

It has been shown before (Arroo, 1994) that *Tagetes* roots which are subcultured in growth medium lacking sulfate, will grow for 14 days at normal rate while at the same time the thiophene concentration drops rapidly. When, on the other hand, the roots are subcultured in medium devoid of nitrogen, they stop growing immediately whereas thiophene content does not change. Furthermore, it was shown by the same author that root elongation and lateral root formation of *Tagetes* roots were not affected at sulfate concentrations low enough to cause a marked reduction of thiophene content.

In this chapter further data are presented which indicate that, in the case of low sulfate availability, thiophene synthesis is down-regulated in cultured *Tagetes* roots before effects on primary metabolism are noticeable. Attempts were made to reveal which steps of thiophene synthesis were down-regulated and whether regulation occurred at the level of gene expression.

CHAPTER 5

MATERIAL AND METHODS

Plant material

Plants of *T. patula* L. cv. "Nana" and *T. erecta* L. were grown in a growth chamber under a 16-hour photoperiod in vermiculite Half-strength Hoagland solution was administered to the plants every second day.

Hairy root cultures of *T. patula* and *T. erecta*, were obtained by transformation of *in vitro* grown seedlings with *Agrobacterium rhizogenes* LBA9402 containing the pRi 1855 plasmid (Birot *et al.* 1987), according to a previously described procedure (Croes *et al* 1989[b]). The transgenic nature of the root clones was ascertained by their ability to grow *in vitro* on hormone-free medium, opine detection, Southern blot hybridization using the Eco RI-15 fragment of pRi 1855 (Birot *et al.*, 1987) as a probe, and PCR amplification of part of the *rol* C gene (not shown). Hairy roots were subcultured in 100-ml Erlenmeyer flasks containing 20 ml hormone-free growth medium consisting of Gamborg's B5 salts (Gamborg, 1970, except for Na_2SO_4) supplemented with 3 % (w/v) sucrose and 100 $\mu g.l^{-1}$ biotin, starting with 10 root tips of 1 cm per flask. The medium was prepared to contain either 2 0 mM ("normal") or 50 μM ("low") sodium sulfate. The flasks were incubated in the dark at 25 °C on a rotary shaker at 100 rpm. In experiments designed to study recovery of thiophene synthesis, roots were cultured for 10 days in medium containing either 2.0 mM or 50 μM sulfate, and subsequently transferred to fresh medium containing 2.0 mM sulfate and, in some experiments, 5 $\mu g.ml^{-1}$ cordycepin.

Thiophene analysis

Plant material was extracted as described earlier (Croes *et al.*, 1989[a]) Thiophenes were recovered from the apolar fraction, and subsequently separated by HPLC on a RP-18 column (250 x 4 mm, 7 μm particle size) with acetonitril:water (70:30) as an eluent. The absorption of the eluate was monitored at 340 nm and the thiophene concentrations were calculated on the basis of the molar absorption coefficients.

Thiophene synthesis, sulfate uptake and sulfate concentration

$Na_2{}^{35}SO_4$ (37 TBq.mmol^{-1}) was purchased from Amersham. Cultured roots in medium containing either 2 0 mM or 50 μM sulfate were labeled with [^{35}S]sulfate (74 kBq.ml^{-1}) and at the same time the sulfate concentration in the medium was raised to 30 mM to minimize label dilution by the internal sulfate pools of the roots. After 4 hours of incubation under standard conditions, the roots were rinsed twice with ice-cold 100 mM Na_2SO_4. The fresh weight was determined and thiophenes were extracted Samples of the polar and apolar extraction phases were mixed with scintillation fluid and counted in a liquid scintillation counter. The total radioactivity in the root was used as a measure for sulfate uptake The radioactivity in the organic phase of the extract was used to estimate thiophene synthesis. Previous HPLC analysis had shown that over 95 % of the ^{35}S counts in the organic phase are in thiophenes.

The sulfate concentration in the medium was determined by culturing roots in growth medium containing either 2.0 or 50 μM sulfate to which a trace amount of $Na_2{}^{35}SO_4$ was added. Samples were taken from the medium at regular intervals for 12 days and radioactivity was counted The amount of radioactivity in the samples was used to calculate the remaining sulfate concentration in the medium

Preparation of radioactive thiophenes

Radioactive thiophenes (BPT, BBT, and MeBBT) were obtained by incubating young plants for several days in 10 ml half-strength Hoagland solution containing 15 MBq of $^{35}SO_4{}^{2-}$ per plant (specific activity 750 MBq.mmol^{-1}). Thiophenes were extracted from roots and hypopocotyls as described and purified by subjecting highly concentrated extracts to preparative HPLC on a Lichrosorb RP-18 column with acetonitrile:water (65·35) as the eluent. Selected fractions were pooled, the eluent was evaporated under nitrogen gas and the purified thiophenes were dissolved in ethanol. Purity of the isolated compounds was confirmed by analytical HPLC.

Thiophene conversions

Three ^{35}S labeled thiophenes were added separately to hairy roots which had been cultured for 10 days in media containing 2 0 mM and 50 μM sulfate (BPT: 18 nmol per flask, at 150 Bq nmol^{-1} specific activity, MeBBT: 7 nmol per flask, at 400 Bq.nmol^{-1}; BBT: 22.5 nmol per flask, at 90 Bq nmol^{-1}). All incubations were carried out in duplicate After 16 hours, thiophenes

were extracted from the roots. Distribution of radioactivity over various thiophenes was determined by separating the extracts on an HPLC column, collecting the eluate in 75 fractions of 0.5 ml per run, and counting the radioactivity in all fractions by liquid scintillation counting.

In vitro translation

Total RNA was extracted from cultured roots using a phenol/chloroform extraction procedure. Four μg of total RNA was translated using the RPN.1 wheat germ translation system (Amersham) and ^{35}S-labeled methionine (Amersham) according to the manufacturers instructions. The radiolabeled proteins were precipitated with trichloroacetic acid, and electrophorized in a 0.1% SDS/10% polyacrylamide gel alongside of radiolabeled molecular weight standards. The gel was dried and exposed to an X-ray film.

Non-protein thiol determinations

The non-protein thiol content was determined according to De Knecht *et al* (1992). Approximately 20 mg lyophilized roots were homogenized at 0°C in 2 ml of a solution containing 200 mM 5-sulfosalicylic acid and 6 3 mM diethylenetriaminepentaacetic acid. The homogenate was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was immediately assayed for sulfhydryl groups by mixing 300 μl with 630 μl 500 mM K_2HPO_4 and 25 μl 10 mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), at a final pH of 7 5, and measuring the absorbance at 412 nm.

RESULTS

To study the effects of sulfate limitation on primary and secondary metabolism, roots of *T. patula* and *T. erecta* were grown in media containing either 2.0 mM ("normal") or 50 μM ("low") sulfate. After 10 days of culturing in low-sulfate medium, thiophene content was reduced by 35% for *T. patula* and 55% for *T. erecta*, whereas the amount of biomass and the protein content were not affected by sulfate limitation (Table 5.1). The thiophene synthesizing capacity of the roots cultured in low-sulfate medium was at least ten times lower than

that of the control roots. This could not be attributed to differences of the capacity to absorb sulfate which was comparable or even higher for roots cultured in low-sulfate medium (Table 5.1).

Table 5.1 Characterization of root cultures grown for 10 days on medium containing 50 μM or 2.0 mM sulfate.

	T. patula		*T. erecta*	
Sulfate concentration	50 μM	2.0 mM	50 μM	2.0 mM
Fresh Weight (g)	0.25 ± 0.02	0.29 ± 0.05	0.30 ± 0.02	0.27 ± 0.03
Protein ($mg.gFW^{-1}$)	0.13 ± 0.01	0.12 ± 0.01	n.d.	n.d.
Thiophenes ($\mu mol.gFW^{-1}$)	0.7 ± 0.2	1.1 ± 0.1	0.5 ± 0.2	1.1 ± 0.2
Sulfate uptake ($\mu mol.gFW^{-1}.h^{-1}$)	1.80 ± 0.03	1.0 ± 0.01	2.5 ± 0.04	2.2 ± 0.03
Thiophene synthesis ($nmol.gFW^{-1}.h^{-1}$)	1.1 ± 0.4	16.7 ± 0.9	0.5 ± 0.1	14.7 ± 2.7

n.d.: not determined.

The at least tenfold difference in thiophene synthesizing capacity concommitant with an approximately twofold difference in thiophene content between roots cultured in low- and normal-sulfate medium, suggested that the thiophene synthesizing capacity not immediately but gradually decreased in case of limited sulfate availability. To investigate this, roots were cultured in media containing either 2 mM or 50 μM sulfate and the capacity to synthesize thiophenes as well as other parameters of primary and secondary metabolism were measured at regular intervals (Fig. 5.1). Again, sulfate limitation had no

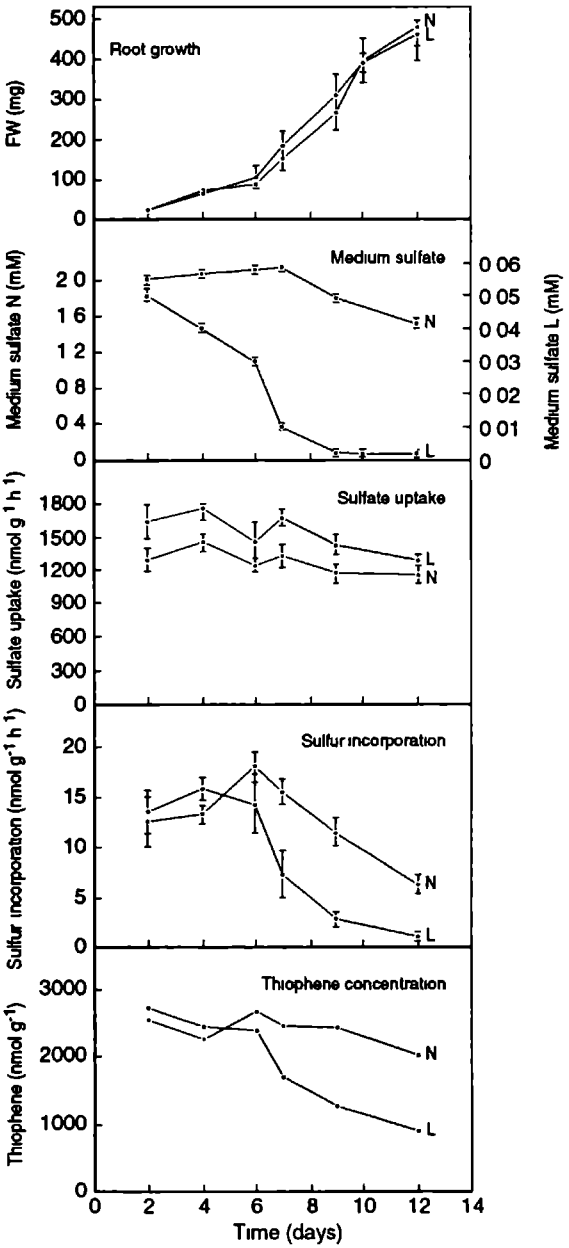

Figure 5.1 Metabolic characteristics of a *Agrobacterium rhizogenes* transformed root culture growing in media containing either 2 0 mM (N) or 50 μM (L) sulfate

effect on the growth rate of the roots during at least twelve days. In the low-sulfate medium, 96% of the sulfate was taken up within eight days, whereas in the normal-sulfate medium only 25% had been absorbed by the roots after 12 days. The capacity to synthesize thiophenes at low sulfate supply started to decrease only after six days. The decrease was fairly gradual and was, as expected, accompanied by a slower decrease in thiophene content. The thiophene synthesizing capacity of normally cultured roots decreased at a later stage and more slowly. This had only a minor effect on thiophene content within the investigated period. The data confirmed that the roots did not immediately reduce their thiophene synthesizing capacity in response to the sulfate limitation. Nevertheless, thiophene synthesis was affected by the low sulfate concentration at least six days before effects on root growth rate were apparent.

The reduction in thiophene synthesizing capacity in case of sulfur limitation could be the result of an active regulatory process involving reduced enzyme synthesis or enzyme inactivation. Alternatively, reduced thiophene synthesis might be due to shortage of the donor compound from which the sulfur in the thiophene ring is directly derived. In the latter case, the concentration of non-protein thiols would be expected to decrease in case of sulfate limitation, whereas the non-sulforous polyacetylenic precursors of thiophenes should accumulate. To test this, the non-protein thiol and polyacetylene content of roots cultured for 10 days in low- and normal-sulfate medium were determined. The concentration of non-protein thiols in low-sulfate roots (0.20 ± 0.04 μmol.g FW^{-1}) was comparable to that in control roots (0.18 ± 0.03 μmol.g FW^{-1}). Also, no increase in the number or amount of polyacetylenes was detected by HPLC analysis (data not shown). The results indicated that shortage of the sulfur donor was not a likely cause of reduced thiophene synthesis under sulfate limitation, leaving the alternative explanation of active regulation the most probable one.

Evidence in support of active regulation was obtained in an experiment designed to determine the time course of recovery of thiophene synthesis when

sulfate supply was restored from a low to the normal level. The thiophene synthesizing capacity was determined at time intervals, of roots that were transferred to fresh normal-sulfate medium after having been pre-cultured for ten days in low-sulfate medium (Fig. 5.2). Recovery did occur, but only after a lag period of at least 8 hours. The delayed onset of recovery argued in favor of active regulation involving mRNA and/or enzyme synthesis. To test the hypothesis of molecular regulation, the recovery experiment was repeated in the presence of an inhibitor of poly(A)$^+$ mRNA maturation (Fig. 5.3). Indeed, it was found that recovery did not occur in the presence of cordycepin at 5 μg.ml^{-1}, a concentration that reduced root elongation to 70 % of the normal rate within one day and to zero after three days (not shown). The thiophene synthesizing capacity of control roots decreased to 60 % of the initial level within one day after transfer to cordycepin-containing medium. This indicated that continuous mRNA synthesis was required to maintain the normal thiophene synthesizing capacity.

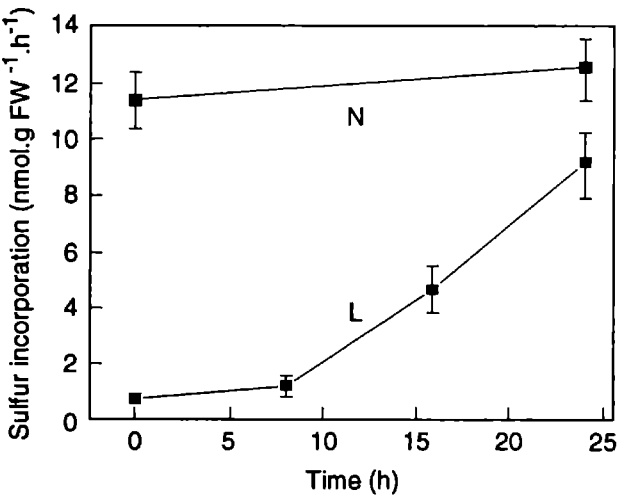

Figure 5.2 Recovery of thiophene synthesis upon transfer of roots to fresh medium containing 2.0 mM sulfate. Roots were precultured for 10 days in media containing either 2.0 mM (N) or 50 μM (L) sulfate.

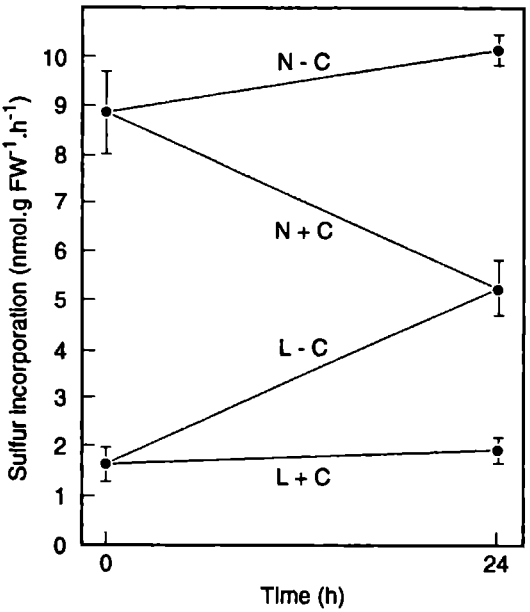

Figure 5.3 Recovery of thiophene synthesis upon transfer of roots to fresh medium containing 2 mM sulfate and cordycepin at 5 μg.ml^{-1} (+C) or without cordycepin (-C). Roots were precultured for 10 days in media containing either 2.0 mM (N) or 50 μM (L) sulfate.

An attempt was made to visualize differences in mRNA production that might be related to the sulfur status of cultured roots. For this purpose, RNA was isolated from roots that had been cultured in media containing either 2.0 mM or 50 μM sulfate and from roots that had been allowed to recover from sulfate limitation for one day. The RNA was translated *in vitro* in the presence of [^{35}S]methionine and the translation products were separated by electrophoresis. No differences were detected among the translation products of mRNAs originating from the differently cultured roots (Fig. 5.4).

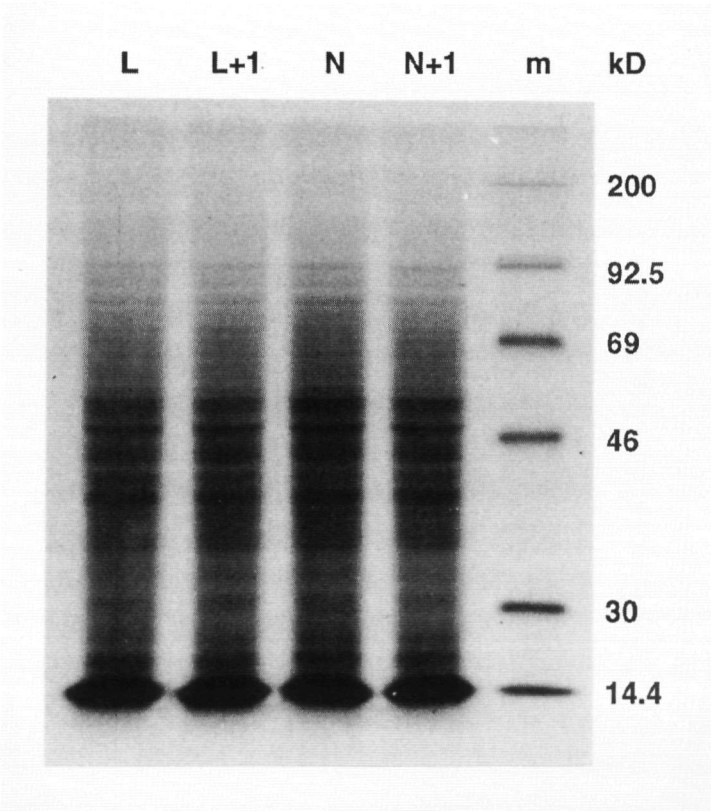

Figure 5.4 Polyacrylamide gel analysis of radioactively labeled *in vitro* translation products obtained from total RNA of roots cultured for 10 days in media containing either 2.0 mM or 50 μM sulfate (N and L, respectively), and from roots cultured in the same media that were subsequently cultured for one day in fresh medium containing 2 mM sulfate (N+1 and L+1, respectively). The m-lane contains molecular weight marker-proteins.

Another line of inquiry was aimed at revealing at the metabolic level which steps of thiophene synthesis were down-regulated in case of sulfate limitation. To this end, feeding experiments were conducted with three ^{35}S-labeled thiophenes. Conversion of the monothiophene BPT to its normally occurring bithiophene products requires incorporation of sulfur in the second

thiophene ring. Conversion of MeBBT and BBT to their major products merely requires modification of the side chains and does not involve reactions utilizing sulfur. If, in the case of sulfate limitation, only those reactions were down-regulated in which sulfur is incorporated, then conversion of BPT should be inhibited whereas conversion of MeBBT and BBT should not be affected. It was found, however, that the conversion rates of all three thiophenes were significantly reduced in roots cultured for 10 days in medium containing 50 μM sulfate (Table 5.2). The reduction was most pronounced for the conversion of BPT to bithiophenes which indicated that this conversion was more severely inhibited than modification of bithiophenes.

Table 5.2 Thiophene bioconversions in roots cultured in growth medium containing either 2.0 mM or 50 μM sulfate

% radioactivity in compounds	radioactive thiophene supplied					
	BPT		MeBBT		BBT	
	2.0 mM	50 μM	2.0 mM	50 μM	2.0 mM	50 μM
BPT	**13.5±3.0**	**62.4±0.5**				
BBT	35.3±1.3	24.9±0.5			**52.7±5.7**	**67.7±0.1**
BBTOH					9.8±2.2	3.5±0.4
BBTOAc	24.8±1.0	6.3±0.1			24.3±6.3	22.5±0.1
BBT$(OAc)_2$					8.2±1.6	6.3±0.2
MeBBT	16.9±3.3	3.4±0.2	**56.2±3.4**	**73.8±2.9**		
$AcOCH_2BBT$	9.6±2.0	3.2±0.0	25.8±0.1	14.6±1.7		
unidentified			18.3±1.3	11.7±0.3	5.0±1.0	0.0±0.0
all products	86.5±3.0	37.7±0.4	43.9±3.4	26.3±2.9	47.3±5.7	32.3±0.1

Bold printing indicates the % of radioactivity remaining in the substrate supplied.

DISCUSSION

The data presented in this chapter indicate that thiophene synthesis is at least partially regulated at the transcriptional level. It is proposed that a regulatory mechanism responds to sulfate shortage by specifically reducing the expression of genes involved in thiophene synthesis before primary metabolism is affected. It is presently not known which genes are less expressed in case of sulfate shortage. Analysis of *in vitro* translation products of mRNAs from roots that had been cultured at different sulfate concentrations failed to reveal major differences between the roots at the transcriptional level. At the metabolic level, it was shown that both early and late steps of thiophene synthesis are down-regulated under sulfate limiting conditions. However, the formation of the second thiophene ring, a conversion that requires sulfur incorporation, was much more reduced than thiophene modifications in which no sulfur incorporation is involved.

Conditions of sulfate limitation did not influence growth and development of *Tagetes* roots during a period of at least twelve days, nor the protein content and the relative abundance of major translatable mRNAs. A further indication that primary metabolism was not affected by the low sulfate concentration comes from the observation that the sulfate uptake capacity only increased slightly in case of sulfate limitation. In other investigations, it was shown that the sulfate uptake capacity increased tenfold over a ten-day period of sulfur deprivation (Hawkesford and Belcher, 1991). Inhibitory effects on thiophene synthesis on the other hand, where noticeable already after seven days. After ten days, both the overall biosynthetic rate and the efficiency of specific bioconversions were reduced. The combination of continuous growth with reduced thiophene synthesis indicated that a mechanism exists which induces down-regulation of thiophene synthesis in favor of primary metabolism in case of sulfate shortage. Determinations of the non-protein thiol content indicated that shortage of the sulfur donor used in thiophene ring formation is not likely the cause of reduced thiophene synthesis. This is further substantiated by the

fact that both sulfur requiring and non sulfur requiring reactions of thiophene synthesis are down-regulated in case of sulfur limitation.

Alternative models to explain the reduced thiophene synthesis under sulfate-limiting conditions have in common a reduced activity of biosynthetic enzymes. This may be accomplished by enzyme inactivation or by a shifting of the balance between enzyme synthesis and enzyme degradation resulting in a lower steady-state enzyme concentration. Evidence in favor of regulation at the level of enzyme activity was obtained from the recovery experiments. The gradual increase of thiophene synthesis following an initial non-responsive period upon transfer to non-limiting sulfate conditions indicated that replenishment of the sulfate pools was not sufficient to effect recovery. The complete absence of recovery in the presence of an inhibitor of RNA polyadenylation indicated that gene expression was required for recovery of thiophene synthesis.

Transcriptional regulation of genes by sulfur nutritional stimuli has been observed before (Fujiwara *et al.*, 1992). This type of regulation is most effective if the encoded enzymes are relatively labile. The reduction of thiophene synthesizing capacity after exposure of control roots to cordycepin for one day indicates that continuous mRNA synthesis is required to maintain a certain level of thiophene synthesis, which implies that (some) enzymes of thiophene synthesis are quite unstable. The amount available of these enzymes may be regulated efficiently by modulation of the transcriptional activity of the coding genes.

The feeding experiments clearly showed that various steps of thiophene synthesis, from formation of the second thiophene ring onwards are down-regulated by sulfur-limitation. The failure of polyacetylenes or BPT to accumulate in case of sulfate shortage suggests that also earlier steps of thiophene synthesis (polyacetylene-formation and first ring-formation) are down-regulated. Moreover, the fact that the overall rate of thiophene synthesis (S-incorporation; Table 5.1) seems to be much more strongly reduced by sulfate limitation than the efficiency of second ring formation, hydroxylation, and

acetylation (Table 5.2) indicates that the limiting step of thiophene synthesis under sulfate limitation may be the formation of either the first thiophene ring, or of the polyacetylenic precursor of thiophenes. Since formation of the first thiophene ring is the first committed step of thiophene synthesis and the first sulfur-requiring step, it would be an appropriate target for major regulatory control. Another argument in favor of first ring-formation being the limiting and most regulated step of thiophene biosynthesis resides in the fact that no monothiophenes accumulate in rots of *Tagetes* plants.

Formation of the second thiophene ring was more efficiently down-regulated than hydroxylation and acetylation of thiophenes. Since sulfate shortage is the primary trigger for down-regulation of thiophene synthesis, it is not surprising that a sulfur-requiring step, formation of the second thiophene ring, is down-regulated more efficiently than non sulfur-requiring steps. Thus, albeit not to the same extent, sequential steps of the thiophene biosynthetic pathway seem to be regulated concertedly. In several instances, concerted regulation of a number of steps of one secondary pathway has been reported to occur at the transcriptional level (Ozeki *et al.*, 1990; Kubasek *et al.*, 1992; Van der Meer *et al.*, 1992). Such concerted transcriptional regulation may operate in the *Tagetes* system as well.

CHAPTER 6

CONCERTED GENE EXPRESSION IN RELATION TO THIOPHENE SYNHTESIS IN *TAGETES* SPECIES

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ABSTRACT

To study at the molecular level the relation between the differentiated state and the activity of secondary metabolism in roots and hypocotyls, a cDNA library was constructed from poly(A)$^+$ RNA of a thiophene-producing root culture of *Tagetes patula*, and differentially screened against a cDNA probe prepared from poly(A)$^+$ RNA of leaves, which are low in thiophenes. Six cDNA clones were isolated and partially sequenced. The accumulation levels of the corresponding or related transcripts were determined in organs and cultured roots of *T. patula* and other plants. One clone, corresponding to a polyubiquitin transcript, revealed expression of ubiquitin genes in all tissues analyzed. The deduced peptides of three cDNA clones showed significant homology to members of the *Betvl* family of pathogenesis-related proteins. Genes related to these clones were strongly expressed in roots of *T. patula* and *T. erecta*, but not in hypocotyls and leaves. Transcripts related to two clones which showed no homology to known sequences were present in roots and hypocotyls but not in leaves of both *Tagetes* species. Since thiophenes predominantly accumulated in roots and hypocotyls, it was concluded that at the organ level the genes corresponding to the latter two clones are expressed as may be expected for genes involved in thiophene synthesis. However, the accumulation levels of transcripts detected by the latter two clones did not reflect the differences in thiophene content and thiophene synthesizing capacity between roots, hypocotyls and various root cultures of both *Tagetes* species.

INTRODUCTION

Secondary metabolites perform a great variety of important functions in plants (Harborne, 1988) which justify the input of energy and substrates in their synthesis. To minimize costs and adverse effects, the synthesis of secondary compounds is strictly regulated and usually occurs only in specific differentiated

organs in a developmentally controlled manner or as a response to environmental signals (Wiermann, 1981). The establishment of secondary metabolism as well as the process of organ differentiation are principally regulated at the molecular level (Hahlbrock and Scheel, 1989). For these reasons, the gene expression which is required for secondary compound synthesis usually coincides with the expression of genes involved in differentiation. The incapability in many cases of undifferentiated cultured cells to synthesize secondary compounds (Rhodes *et al.*, 1987; Kreis and Reinhard, 1988) argues for a coordinated regulation of secondary metabolism and differentiation. That is, common regulatory signals and mechanisms elicit the gene expression that is required both for differentiation and for secondary metabolism. This coordinated regulation apparently does not occur when secondary compound accumulation is elicited in already differentiated organs or in undifferentiated cell cultures by specific treatments (Gleitz *et al.*, 1991; Kawalleck *et al.* 1992). In these cases, the expression of genes of secondary metabolism is not linked to the expression of differentiation related genes.

Various Asteraeceous species contain nematicidal compounds known as thiophenes (Uhlenbroek and Bijloo, 1958; 1959). These compounds are synthesized *in vivo* from polyacetylenic precursors, which in turn are derived from poly-unsaturated fatty acids (Bohlmann *et al.* 1973; Bohlmann and Zdero, 1985). The biosynthetic pathway of thiophenes has been poorly characterized and no enzymes involved in thiophene biosynthesis, let alone the corresponding genes have been isolated.

Tagetes patula and *T. erecta*, commonly known as French and African marigolds respectively, synthesize and accumulate the same group of thiophenes predominantly in the roots and in the lower part of the stem throughout the vegetative growth phase (Sütfeld, 1982; Downum and Towers, 1983; Mukundan and Hjortso, 1990; Jacobs *et al.* , in press). In detached cultured roots and in *Agrobacterium rhizogenes*-transformed hairy roots of *T. patula* and *T. erecta*, thiophene accumulation proceeds during the entire period of exponential growth (Croes *et al.*, 1989[b]; Mukundan *et al.* 1990). It may be

expected that genes encoding enzymes of thiophene synthesis are expressed predominantly in roots and hypocotyls of plants and in exponentially growing cultured roots whereas expression would be low in leaves.

The *Tagetes* system represents an interesting model to study patterns of gene expression in secondary metabolism in relation to differentiation. Firstly, the organs of the related species *T. patula* and *T. erecta* differ considerably with respect to thiophene content and rate of synthesis (Jacobs *et al.*, in press; Chapter 2 of this thesis). Moreover, a mutant of *T. erecta* is available with an altered thiophene spectrum due to the inability to demethylate the C_{13} monothiophene BPT (Chapter 3 of this thesis). Secondly, *Agrobacterium rhizogenes*-transformed root cultures of *T. patula*, *T. erecta* and the mutant *T. erecta* have been established which can be subcultured easily and indefinitely, and which vary considerably with respect to thiophene content (Chapter 3 and 5 of this thesis). In these cultured roots the thiophene synthesizing capacity can be accurately quantified, and also can be modulated by changing the sulfate concentration in the growth medium (Arroo, 1994; Chapter 5 of this thesis). Thirdly, specific thiophene bioconversions can be elicited by adding fungal cell wall extracts to cultured roots (Arroo, 1994). The various organs and root cultures that differ with respect to thiophene synthesis, provide a tool to investigate whether or not, and to what extent certain genes are regulated in concert with genes of thiophene synthesis.

As a first step in studying patterns of coordinated gene expression, a cDNA library was constructed using poly(A)$^+$ RNA of an exponentially growing transgenic root culture of *T. patula*. From this library, cDNA clones were isolated by differential screening, and characterized with respect to the accumulation levels of the corresponding or related mRNAs in different organs of *T. patula* and *T. erecta*, and a related (*Bidens alba*) as well as an unrelated (*Lycopersicon esculentum*) species. The accumulation levels of mRNAs were also investigated in *T. patula* hairy roots cultured in media containing different sulfate concentrations, and in hairy roots of wild-type *T. erecta* and a mutant *T. erecta* having an altered thiophene metabolism. The accumulation levels of the

mRNAs were compared to the thiophene content or synthesizing capacity of the respective organs and cultured roots.

MATERIAL AND METHODS

Plant material

Plants of *Tagetes patula*, *T erecta*, *Bidens alba*, and *Lycopersicon esculentum* were grown during the summer in a greenhouse under daylight in vermiculite until the age of three weeks. Half-strength Hoagland solution was administered to the plants every second day

Hairy root cultures of *T. patula*, *T. erecta*, and of a mutant *T. erecta* which accumulates C_{13} thiophenes not found in the wild type, were obtained by transformation of *in vitro* grown seedlings with *Agrobacterium rhizogenes* LBA9402 containing the pRi 1855 plasmid (Birot *et al.* 1987), according to a previously described procedure (Croes *et al* 1989[b]). The transgenic nature of the root clones was ascertained by their ability to grow *in vitro* on hormone-free medium, by opine detection, by Southern blot hybridization using the Eco RI-15 fragment of pRi 1855 (Birot *et al* , 1987) as a probe, and by PCR amplification of a part of the Eco RI-15 fragment (not shown). The hairy roots were subcultured in Erlenmeyer flasks containing 20 ml hormone-free growth medium consisting of Gamborg's B5 salts (Gamborg, 1970) supplemented with 3 % (w/v) sucrose and 100 $\mu g\ l^{-1}$ biotin, starting with 10 root tips of 1 cm per flask. The flasks were incubated in the dark at 25 °C on a rotary shaker at 100 rpm. In some experiments, the sulfate concentration in the growth medium was lowered from 2.0 to 0.05 mM. When cultures were treated with fungal cell wall extract, the extract was added to the roots after 8 days of culturing in B5 medium and the roots were harvested 24 hours later. In these experiments an equal volume of water was added to control roots.

Fungal culture and elicitor preparation

Strain F14 of *Fusarium oxysporum* was kindly supplied by the Department of Microbiology of the Wageningen Agricultural University. The fungus was routinely maintained on 1 % (w/v) soytone (Difco) solidified with 1 % gellan gum (Gelrite) in 9-cm Petri dishes at 25 °C in the dark. For elicitor preparation, a 5-mm agar disc from a full-grown culture was used to inoculate 50 ml of 1 % (w/v) soytone in a 300-ml Erlenmeyer flask After 14 days of growth in the dark

without shaking, the mycelium was harvested, washed and resuspended in demineralized water, and homogenized. The homogenate was filtered over a Whatman GF/C filter and the carbohydrate content of the filtrate was determined (Miller, 1959). The filtrate was lyophilized, stored as a freeze-dried preparation, dissolved in water and autoclaved before use.

Thiophene analysis

Plant material was extracted as described earlier (Croes *et al.*, 1989a). Thiophenes were recovered from the apolar fraction, and subsequently separated by HPLC on a RP-18 column (250 x 4 mm, 7 μm particle size) with acetonitrile:water (70:30) as an eluent. The absorption of the eluate was monitored at 340 nm and the thiophene concentrations were calculated on the basis of molar absorption coefficients.

Thiophene synthesizing capacity

$Na_2{}^{35}SO_4$ (37 GBq.μmol^{-1}) was purchased from Amersham Exponentially growing root cultures in medium containing either 2.0 mM or 50 μM sulfate were labeled with ^{35}S-sulfate (0.74 MBq.ml^{-1}) At the same time the sulfate concentration in the medium was raised to 30 mM to minimize label dilution by the internal sulfate pools of the roots. After 4 hours of incubation under standard conditions, the roots were rinsed twice with ice-cold 100 mM Na_2SO_4. The fresh weight was determined and thiophenes were extracted Samples of the polar and apolar extraction phases were mixed with scintillation fluid and counted in a liquid scintillation counter. The total radioactivity in the root was used as a measure for sulfate uptake. The radioactivity in the organic phase of the root extract was used to estimate thiophene synthesis. Previous HPLC analysis had shown that over 95 % of the ^{35}S counts in the organic phase are in thiophenes.

Preparation of radioactive BBT

Radioactive BBT was obtained by incubating young plants for several days to one week in growth medium containing 15 MBq of ^{35}S-labeled sulfate (Amersham) per plant. Thiophenes were extracted as described and purified by subjecting highly concentrated extracts to preparative HPLC on a Lichrosorb RP-18 column with acetonitrile.water (65:35) as an eluent. Selected fractions were pooled, the eluent was evaporated under nitrogen gas and the purified

thiophenes were dissolved in ethanol Purity of the isolated compounds was confirmed by analytical HPLC.

Thiophene conversion

^{35}S-labeled BBT (specific activity 90 Bq nmol^{-1}) was added to hairy roots which had beer cultured for one day in the presence of fungal elicitor and to control hairy roots (22 5 nmol per flask). After 24 hours thiophenes were extracted from the roots and from the growth medium. Distribution of radioactivity over various thiophenes was determined by separating the extracts on an HPLC column, collecting the eluate in 75 fractions of 0 5 ml per run, and counting the radioactivity in all fractions by liquid scintillation counting.

RNA extraction

Total RNA was extracted from nine-days-old hairy root cultures and from roots, hypocotyls and leaves of three-weeks-old plants using a phenol/chloroform extraction procedure. Poly(A)$^+$ RNA was purified from total RNA samples by separation on an oligo(dT)-cellulose column (Sambrook *et al.*, 1989).

Library construction and differential screening

A directional cDNA library was constructed from 10 μg poly(A)$^+$ RNA isolated from a nine-days old exponentially growing *Agrobacterium rhizogenes*-transformed hairy root culture of *T patula*, using a Uni-ZAP XR cDNA synthesis kit and Gigapack II Gold packaging extracts (Stratagene) according to manufacturer's instructions. The library was amplified once before screening.

Duplicate plaque lifts of the library were prepared using nitrocellulose membranes. The membranes were hybridized with [^{32}P]dATP-labeled single-strand DNA probes prepared from poly(A)$^+$ RNA isolated from nine-days-old *T. patula* hairy roots (second membrane) and from leaves of three-weeks-old *T patula* plants (first membrane). Selected recombinant phages were isolated and subjected to a second round of hybridization screening using the same probes.

The ExAssist/SOLR *in vivo* excision system of Stratagene was used for automatic excision of selected cDNA clones from lambda ZAP II to yield the insert-containing pBluescript II SK(-) vector in XL-1 Blue *E coli* cells

The size of the inserts of 63 plasmid clones was determined by restriction digestion with Eco RI and Xho I and subsequent electrophoresis in a 0.8 % agarose gel. The clones were placed in cross-hybridizing groups by Southern blot hybridization of Hybond-N membranes (Amersham) containing digested and electrophorezed DNA from all plasmid clones to [^{32}P]dATP-labeled inserts of randomly selected clones.

Preparation of hybridization probes and sequence analysis

Plasmid DNA was prepared as outlined in Sambrook *et al.* (1989). Radioactively labeled probes for Southern and Northern blot hybridization were prepared from cDNA fragments in low-melting point agarose using the random-primer labeling method (Feinberg and Fogelstein, 1983).

Nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the T7 DNA polymerase sequencing system of Pharmacia. Sequences were analyzed using the University of Wisconsin Genetics Computer Group programs (Devereux *et al.*, 1984). Homology searches were performed using the program FASTA to search the databases Pyr and Swiss.

Northern blot analysis

For Northern blot analysis, 10 μg of total RNA was denatured, subjected to electrophoresis in 1.5 % agarose gels containing 0.4 M formaldehyde, and transferred to Hybond-N (Amersham) by capillary blotting. The RNA was fixed on the membranes by baking for 1 hour at 80 °C. The filters were hybridized to [^{32}P]dATP-labeled probes that were prepared from cDNA inserts. Hybridization was performed overnight at 55 °C according to Sambrook *et al.* in a hybridization oven. Filters were washed twice for 30 minutes at 55 °C in 2x SSC, 0.1% SDS and 1x SSC, 0.1 % SDS. Filters were exposed to Valca HPX44 X-ray films with an intensifying screen at -80°C. For reuse, filters were stripped by rinsing in 0.1x SSC at 95 °C for one minute.

RESULTS

Library construction

An oriented cDNA library was constructed from poly(A)$^+$ RNA isolated from a nine-days-old root culture of *T. patula* transformed by *A. rhizogenes* LBA9402 (pRi 1855; Birot *et al.*, 1987). At the time of RNA extraction the roots had a thiophene synthesizing capacity of 10 nmol.g^{-1}.h^{-1}, and the growth rate and thiophene content were high (Fig. 6.1). The primary library consisted of 276,500 plaques. The majority of phages (91 %) contained an insert, as was indicated by their inability to convert 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) to a blue-colored product. After *in vivo* excision of plasmids from 18 randomly selected phages, plasmid DNA was isolated and analyzed by restriction enzyme digestion and gel electrophoresis. Fifteen plasmid clones contained inserts ranging in size from 200 to 3100 base pairs.

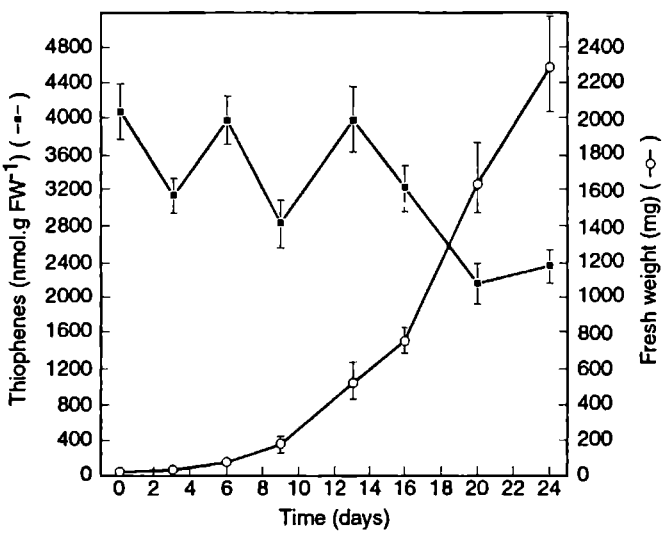

Figure 6.1. Growth and thiophene content of a root culture of *Tagetes patula* transformed by *Agrobacterium rhizogenes* strain LBA9402.

Differential screening

About 30,000 plaques were differentially screened using two radiolabeled cDNA probes. The control probe was derived from poly(A)$^+$ RNA extracted from a nine-days-old transgenic *T. patula* root culture. The other probe was prepared against poly(A)$^+$ RNA extracted from leaves of three-weeks-old greenhouse-grown *T. patula* plants. About 30 % of the plaques gave a hybridization signal of equal strength with both probes. About 2 % of the plaques gave a stronger signal with the root probe and another 2 % gave a stronger signal with the leaf probe. After the first screening, 118 plaques were selected and subjected to a second screening with the same probes. Hereafter 63 plaques which fell into three categories were selected for further analysis. Two plaques hybridized stronger to the leaf probe; 16 plaques that gave about equal, mostly strong, signals with both probes and 45 plaques gave a significantly stronger signal with the root probe. All 63 phage clones were converted to plasmid clones by *in vivo* excision. All plasmids contained an insert, ranging in size from 200 to 1600 base pairs. On Southern blots none of the cDNA inserts hybridized to a radiolabeled Eco RI-15 fragment of the pRi 1855 plasmid (Birot *et al.*, 1987), indicating that none of the clones corresponded to a *Rol*-gene transcript.

Groups of cross-hybridizing clones were identified by radiolabeling randomly selected cDNA inserts and hybridizing them to Southern blots containing digested DNA from all 63 plasmid clones. In this way, 57 clones could be classified in 22 groups. Twelve groups contained only one single clone, whereas nine groups contained 2 to 6 clones. One group contained 19 clones. Six clones were not placed in a group since they did not give a signal with any of the radiolabeled inserts tested.

Sequence data

Six cDNA clones, representing as many cross-hybridizing groups, were partially sequenced from the 5' end. The cross-hybridizing groups from which a representatives was selected exclusively contained clones that, according to the differential screening results, corresponded to mRNAs that accumulated at higher levels in hairy roots than in leaves. A single sequencing run yielded 163 to 296 bases of sequence information. In each sequence an open reading frame of 54 to 86 amino acids identified in the sense orientation was. Nucleotide and deduced peptide sequences were compared to sequences from the Swiss and Pyro data bases using the FASTA program. The results are summarized in Table 6.1.

Table 6.1 Characteristics of partially sequenced cDNA clones

cDNA clone	Insert size (bp)	Cross-hybridizing clones[1)]	Nucleotides sequenced (bp)	Identity to known sequences (amino acid level)
TPC57	1000	1	211	polyubiquitin (100% identity)
TPC87	600	3	296	*Betvl* family of PR proteins (37-39% identity)
TPC89	550	19	230	*Betvl* family of PR proteins (35-40% identity)
TPC137	400	2	163	*Betvl* family of PR proteins (35-43% identity)
TPC44	500	4	241	no homology
TPC128	600	3	220	no homology

[1)] Including the cDNA clone listed in the table.

Tagetes patula cDNA (TPC) 57 showed a high degree of homology to coding regions of polyubiquitin genes (81 - 88 % identity). The deduced peptide of this clone was 100 % identical to regions comprising the C-terminal and N-terminal parts of contiguous ubiquitin units of polyubiquitin proteins of sunflower (Binet *et al.*, 1991), maize (Christensen *et al.*, 1992) and several other plants. Thus it was concluded that TPC57 represents a partial polyubiquitin mRNA of *T. patula*.

TPC87 and TPC89, although belonging to different cross-hybridizing groups, were highly homologous to each other (83 % identity). These clones together with TPC137 showed at the amino acid level significant homology (37-40 % identity) to various members of the *Betvl* family of pathogenesis-related proteins identified in pea (Fristensky *et al.*, 1988), parsley (Somssich *et al.*, 1988; van de Löcht *et al.*, 1990), birch (Breiteneder *et al.*, 1989), bean (Walter *et al.*, 1990; Awade *et al.*, 1991), potato (Matton *et al.*, 1990), soybean (Crowell *et al.*, 1992), and other species. The deduced peptides of TPC87 and TPC89 were homologous to the N-terminal region of these PR proteins. From the alignments it was concluded that the sequence of TPC87 contained 38 base pairs of the 5' untranslated region and the start codon of the corresponding mRNA, whereas the sequence of TPC89 started at what is probably the 3rd, 4th or 5th codon of the corresponding mRNA. The deduced peptide of TPC137 showed homology to an internal region of the PR proteins, not overlapping the region of homology of the TPC87 and TPC89 peptides. The sequence data together with the cross-hybridisation results indicate that the mRNAs corresponding to the three *Betvl*-related cDNA clones are transcribed from three related genes.

Two clones, TPC44 and TPC128, showed no homology to known sequences, neither at the nucleotide, nor at the deduced amino acid level.

Expression patterns

To establish the relationship between gene expression and thiophene synthesis in various organs, the accumulation levels of transcripts corresponding or related to the sequenced cDNA inserts were analyzed in roots, hypocotyls, and leaves of *T. patula* and *T. erecta*, and two other species (Fig. 6.2). *Bidens alba* was chosen as a member of the same family as *Tagetes*. This species contains polyacetylenes, but not the thiophenes derived therefrom (Norton and Towers, 1986). *Lycopersicon esculentum* (tomato) was chosen to represent less related plant species and does not contain polyacetylenes nor thiophenes. For the two *Tagetes* species, the thiophene content was determined in the same organs from which RNA was isolated for Northern blot analysis (Table 6.2).

Table 6.2 Thiophene content of organs of three-weeks-old soil-grown *Tagetes* plants

Organ	Thiophene content (nmol.g FW^{-1})	
	T. patula	*T. erecta*
root	1683.5 ± 182.8	116.7 ± 13.9
hypocotyl	711.7 ± 112.4	170.9 ± 13.8
leaf	51.8 ± 17.5	5.0 ± 1.1

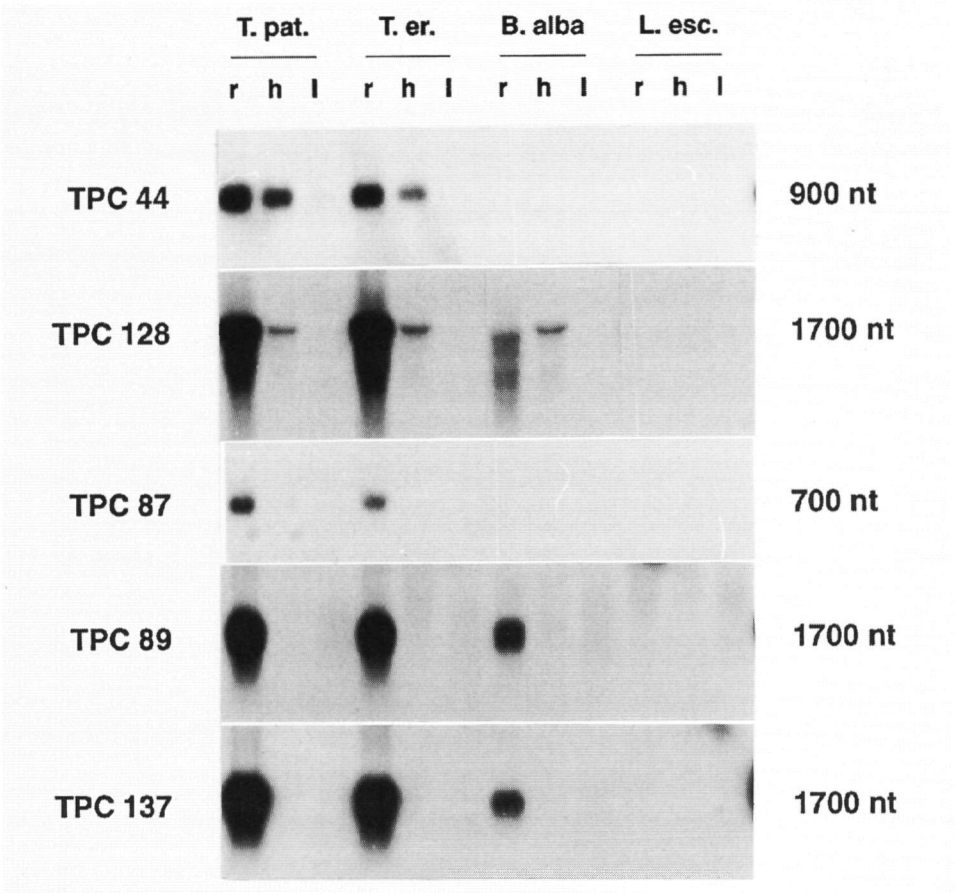

Figure 6.2 Accumulation of transcripts corresponding to five different cDNA clones in roots (r), hypocotyls (h), and leaves (l) of *T. patula*, *T. erecta*, *B. alba*, and *L. esculentum*. RNA gel blots were hybridized to one of five radiolabeled cDNA inserts as marked. An identical blot was hybridized to the radiolabeled ubiquitin cDNA insert, which yielded a clear signal in all lanes. The size of the mRNAs (nucleotides; nt) was estimated by comparison to rRNA bands in the gels.

As expected, a radiolabeled probe of TPC57, corresponding to polyubiquitin mRNA, hybridized to RNA from all organs tested of the four species (not shown). This clone was used in further experiments as a positive control for the

presence of mRNA on the blots. The other five cDNA clones revealed the presence of transcripts in roots and, in the case of two clones, hypocotyls of both *Tagetes* species. In *B. alba* transcripts homologous to three clones were detected in roots and, for one clone, also in hypocotyls. None of the clones except TPC57 hybridized to mRNA from tomato organs or to RNA from *Tagetes* or *Bidens* leaves.

TPC44 and TPC128 were particularly interesting, since transcripts related to these clones accumulated in roots and hypocotyls of both *Tagetes* species, which are also the organs in which thiophenes accumulate at highest levels. In subsequent experiments, the expression of the genes corresponding to these clones was investigated in transformed root cultures of *T. patula* grown at two concentrations of sulfate which caused the thiophene synthesizing capacity to differ by a factor eight (Table 6 3), and in transformed root cultures of wild-type and mutant *T. erecta*. The mutant-derived roots accumulated the lowest amount of thiophenes (Table 6.4), among which a monothiophene and two bithiophenes that are not present in wild-type roots (Chapter 3 of this thesis). It was found that transcripts related to TPC 44 and TPC 128 accumulated to comparable levels in the various root cultures in spite of the significant differences between them with respect to thiophene content, thiophene synthesizing capacity, and thiophene spectrum (Fig. 6.3).

Table 6.3 Sulfate uptake capacity and thiophene synthesizing capacity of transgenic roots of *T patula* cultured for nine days in media containing different amounts of sulfate

Growth medium	Sulfate uptake (nmol $g^{-1}h^{-1}$)	Thiophene synthesis (nmol $g^{-1}h^{-1}$)
B5 / 2 0 mM SO_4^{2-}	1388 6 ± 162 7	4 43 ± 0 67
B5 / 50 μM SO_4^{2-}	1261 4 ± 147 6	0 51 ± 0 04

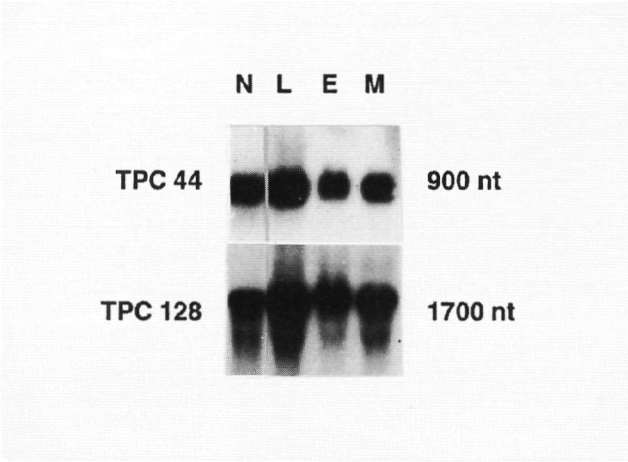

Figure 6.3 Accumulation of transcripts corresponding to TPC44 and TPC128 in various transgenic roots. *T. patula* roots were cultured for 10 days in medium containing either 2.0 mM (N) or 50 μM (L) sulfate. Roots of *T. erecta* (E) and of a mutated *T. erecta* (M) were cultured for 10 days in medium containing 2.0 mM sulfate. A clear signal was observed in all lanes when the ubiquitin cDNA insert was used as a hybridization probe. The size of the mRNAs (nucleotides; nt) was estimated by comparison to rRNA bands in the gels.

Table 6.4 Thiophene content of various *A. rhizogenes* LBA9402-transformed roots of *T. erecta* and *T. patula* cultured for nine days in B5 medium

Root Clone	Thiophene content (nmol.g FW^{-1})
T. patula 9402	3587.4 ± 309.7
T. erecta 9402	1676.3 ± 121.5
T. erecta mutant 9402	927.3 ± 53.1

The three clones that showed homology to *BetvI*-related genes hybridized to transcripts from roots but not hypocotyls of *Tagetes*. Therefore, it could be concluded that the genes corresponding to these clones were not regulated in concert with genes of thiophene synthesis. Because of the homology to *BetvI*-related genes, we investigated whether expression of these genes was enhanced by treatment of roots with a fungal elicitor. Treatment of an exponentially growing *T. patula* root culture with an extract from *Fusarium oxysporum* did promote conversion of BBT to hydroxylated and acetylated derivatives: 90.5% of added BBT was converted within 24 hours in treated roots, whereas only 48.8 % was converted in untreated roots. However, this treatment did not affect the expression of genes related to TPC89 and TPC137 (Fig. 6.4).

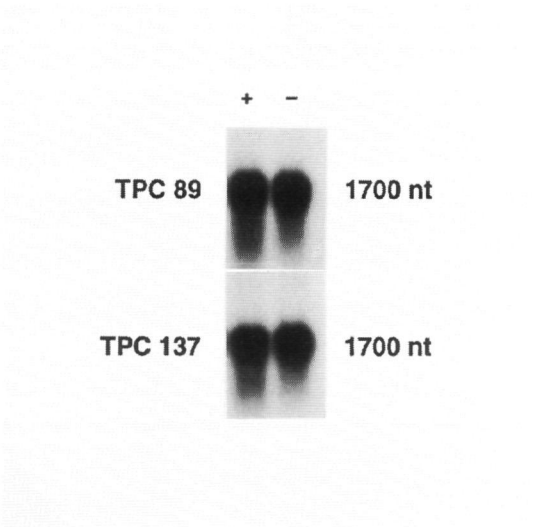

Figure 6.4 Accumulation of transcripts corresponding to TPC89 and TPC137 in transgenic roots of *T. patula* cultured for one day in the presence (+) or absence (-) of a fungal cell wall extract. The roots had been pre-cultured for 10 days before the elicitor was added. A clear signal was observed in all lanes when the ubiquitin cDNA insert was used as a hybridization probe. The size of the mRNAs (nucleotides; nt) was estimated by comparison to rRNA bands in the gels.

DISCUSSION

As a first step in studying the relationship between thiophene metabolism and gene expression in *Tagetes*, we have constructed and differentially screened a *T. patula* hairy root cDNA library. Six cDNA clones were further analyzed by partial sequencing and Northern blot hybridization.

Two cDNA clones are derived from transcripts of genes of unknown function which may, at the organ-level, be regulated in concert with genes of thiophene metabolism. These genes are expressed in roots and hypocotyls of *Tagetes* plants, which are the organs where genes of thiophene synthesis should be expressed because thiophenes are principally synthesized in these organs (Jacobs *et al.*, in press; Chapter 2 of this thesis). Within the roots, the genes seem to be regulated differently from the genes of the rate limiting steps of thiophene synthesis, since the quantitative differences in thiophene content and synthesis between variously cultured roots are not reflected in differences of accumulation levels of the transcripts of both these genes. The expression of the genes corresponding to these two clones was also not altered in mutant *Tagetes* plants (not shown), nor in transgenic root cultures derived thereof (Fig. 6.3), indicating that the mutation(s) did not affect expression of these genes.

The genes corresponding to four other cDNA clones (encoding ubiquitin and *Betvl*-like polypeptides) are not expressed coordinately with genes of thiophene metabolism, beause no transcripts corresponding to these clones were detected in hypocotyls of *Tagetes*.

One clone, TPC57 is highly homologous to coding regions of known polyubiquitin genes. The high degree of homology and the observed expression in all organs are in accordance with expectations, since ubiquitin genes are highly conserved in eukaryotes and are transcribed in all organs of plants.

Three clones show homology to the *Betvl*-family of pathogenesis-related genes at the deduced amino acid level. In *T. patula* the corresponding mRNAs are transcribed from genes of a family of at least three members. PR proteins of the *Betvl* family are usually encoded by a small gene family (Walter *et al.*, 1990;

Crowell *et al.*, 1992). The genes corresponding to the three *Betvl*-related cDNA clones are expressed in roots of *Tagetes* and *Bidens* whereas no expression is observed in hypocotyls and leaves. This parallels the situation in soybean, where mRNAs corresponding to the related SAM22 cDNA clone predominantly accumulate in roots of young seedlings (Crowell *et al.*, 1992). In contrast with the situation in most other species containing *Betvl*-related genes, no induction of gene expression was observed following elicitation of cultured roots. Possibly, in the transgenic root systems that we used, the *Betvl*-related genes are constitutively expressed at high level. The fact that TPC89 was member of a very large cross-hybridizing group also hints in this direction.

It will be interesting to further study the regulation of the genes corresponding to clones TPC44 and TPC128 in relation to thiophene synthesis. However, in future investigations of coordinated gene expression in relation to thiophene synthesis, it will also be crucial to identify genes that are directly involved in thiophene metabolism by a more direct approach.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Secondary metabolism is an increasingly popular area of biochemical research in plants. The relationship between secondary metabolism and structural genes encoding biosynthetic enzymes is straightforward and can be unequivocally established. Plants can often be maintained well even if their secondary metabolism is perturbed by physiological or molecular genetic manipulations. The effects of these perturbations can be measured directly and quantitatively. In addition, many regulatory aspects of secondary metabolism can be studied excellently in cell and organ cultures. These factors make biochemical investigations possible on the function of secondary compounds, and render secondary metabolism a favorite model to study control mechanisms of metabolic processes in general and the regulation of gene expression in particular. Not surprisingly, great advances have been made during the last decade in the fields of both function and regulation of secondary metabolism.

Thiophenes and *Tagetes*, which are the subjects of this thesis, not only present an interesting model for metabolic investigations, but also justify biochemical investigations on their own right. Marigolds have long been known and applied both by commercial farmers and by ecological gardeners for their nematode suppressing properties. The nematode suppressing activity was attributed to thiophenes, which accumulate mainly in the roots of *Tagetes* species and have broad-range biocidal properties. Thiophenes are one of a few classes of sulfur-containing secondary compounds. Biosynthesis of thiophenes from primary metabolites involves at least two types of fairly uncommon and uncharacterized bioconversions. One is the formation of conjugated acetylenic bonds in straight-chain fatty acids. The other is the formation of sulfur-containing, five-membered aromatic rings using conjugated acetylenic bonds as a substrate.

At the outset of this work, the biosynthetic pathway leading to thiophenes had not been studied in great detail in *Tagetes*, nor in other species. None of the presumed enzymes had been characterized biochemically. Thiophene

accumulation in intact plants had been rather poorly characterized, and had not been analyzed in terms of synthesis, transport and degradation. In this thesis new data are presented about various aspects of thiophene metabolism that have led to a better understanding of the regulation of this fascinating secondary metabolic pathway.

Molecular genetic (Chapter 3) and physiological (Chapter 5) manipulations were used to study the pathway of thiophene synthesis and its regulation in intact plants and organ cultures of *Tagetes*. These investigations were preceded by a detailed analysis of the spatial distribution and temporal accumulation patterns of thiophenes (Chapters 2 and 4). Also, the contributions of synthesis and transport to the resultant thiophene accumulation patterns were assessed (Chapter 2). In the final part of the thesis (Chapter 6), a start was made with investigations of thiophene metabolism at the gene level. All taken together, the obtained results contribute to the further development of the *Tagetes*/thiophene system as a suitable model for biochemical and molecular genetic investigations of secondary metabolism.

The major thiophenes of *Tagetes patula* and *T. erecta* are BBT and BBTOAc, and they accumulate predominantly in the roots and the lower part of the stem of both species. Within the hypocotyl, thiophenes are localized predominantly in the epidermis and the vascular tissue. A substantial fraction of the thiophenes is localized intracellularly, at least in the hypocotyl. Also the enzymes of thiophene metabolism are localized intracellularly, since isolated protoplasts are able of thiophene synthesis. On basis of calculations, it is concluded that, at least in the stem, another significant portion of the thiophenes is localized in the extracellular matrix.

Characteristic and distinctive accumulation patterns of individual thiophenes are observed in roots and hypocotyls, and in the two species. Thiophenes accumulate were they are synthesized, since they are poorly transported. The higher thiophene content of *T. patula* is the result of a higher

synthetic activity in roots and stems of this species. The low thiophene content of leaves is not due to limited supply of precursor sulfate, but rather to a very low synthetic activity in these organs. The differences in activity of thiophene metabolism between the two species are not reflected in variations of the expression level of genes corresponding to six selected cDNA clones. The fact that all six cDNA clones reveal identical expression patterns on RNA blots of both species rather indicates that both species are very similar at the molecular level.

In roots as well as hypocotyls, high concentrations of BBT are rapidly established upon germination. This, and the localization of thiophenes in the epidermis and vascular tissue are in agreement with a function in defense against predation during the critical period of early seedling development. Extracellular localization of a major fraction of the thiophenes is not an impediment to their toxicity to predators, but may well be a method to prevent self-toxication. It also explains, at least partially, the limited transport of thiophenes.

Mutants of thiophene metabolism were identified that accumulate C_{13} mono- and biotiophenes which are absent or present in low quantities in wild-type *Tagetes*. Feeding experiments showed for one mutant that it is impaired in the removal of a terminal methyl group from the monothiophene. This monothiophene, BPT, represents a branching point in thiophene synthesis. In one branch, the second thiophene ring is directly formed using BPT as a substrate, leading to MeBBT and its derivatives. In the second branch, the terminal methyl group is first removed and the second thiophene ring is subsequently formed yielding BBT and its derivatives. In wild-type *Tagetes*, the second route predominates strongly. *Tagetes* plants are unable of demethylation once the second ring is formed.

The mutants were of pivotal importance in further investigations, since they provided the evidence that BPT is the monothiophene intermediate in thiophene synthesis. Also, they were an ideal source of ^{35}S-labeled BPT, which was used in feeding experiments described in chapters 4 and 5.

Isolated hypocotyl protoplasts are able to perform all steps of thiophene synthesis, from formation of the first thiophene ring onwards. No factors from the extracellular matrix or neighbouring cells are required for first and second ring-formation, demethylation, hydroxylation, and acetylation to take place. Second ring-formation, hydroxylation, and acetylation occur equally efficiently in leaf blade and hypocotyl protoplasts, irrespective of the fact that the former are from an organ that has a very low thiophene content. It may be concluded that the enzymes of thiophene synthesis are localized intracellularly and that a number of enzymes are active not only in organs containing high thiophene levels but also in organs that are low in thiophenes.

In transgenic root cultures, thiophene metabolism responds to fluctuations of sulfate supply much more sensitively than primary metabolism. Both the overall rate of thiophene synthesis and the rates of several specific bioconversions are reduced in case of sulfate limitation, whereas at the same time primary metabolism is not noticeably affected. Feeding experiments indicate that sulfur-requiring steps are more impeded than non-sulfur-requiring steps in case of sulfate deprivation, and that formation of the first thiophene ring may be the limiting step and most important control point under these circumstances. Recovery of thiophene synthesis upon restoration of normal sulfate levels only occurs after a lag phase and can be completely prevented by addition of an inhibitor of mRNA maturation. This indicates that thiophene synthesis is at least partially regulated at the level of gene expression. No differences in gene transcription could be visualized between sulfur-limited and non-limited roots, neither by *in vitro* translation, nor by Northern blot hybridization.

By differential screening of a *T. patula* cDNA library, two cDNA clones were identified corresponding to genes that are expressed predominantly in roots and hypocotyls of *T. patula* and *T. erecta*, the primary organs of thiophene synthesis. Both clones do not show any significant homology to known genes, neither at the nucleotide, nor at the deduced peptide level. The two clones are interesting because at the organ level they may be regulated in

concert with genes of thiophene synthesis. Within roots and hypocotyls, however, the accumulation levels of the mRNAs corresponding to both cDNAs fail to correlate with thiophene content or biosynthetic activity. This means that within an organ they are regulated differently from genes of thiophene metabolism. The genes corresponding to four other cDNA clones are expressed predominantly in roots of *Tagetes* but not in hypocotyls. The deduced peptides of three of these clones show homology to pathogenesis-related proteins and the fourth is a partial ubiquitin cDNA.

Future research in the *Tagetes*/thiophene system should primarily focus on the identification of enzymes and genes of the thiophene pathway. Only than can the regulation of the pathway be studied at a biochemical level. Several strategies may be adopted to reach this goal, for which the results presented in this thesis provide valuable information and tools.

One option would be to repeat the mutagenesis experiment, using a known transposon or T-DNA sequence to generate mutations. As has been shown in chapter 3, mutants of thiophene metabolism can be obtained at a high frequency. A mutant gene containing a DNA-tag can be cloned relatively easily via one of several procedures. However, a DNA-tagging approach would require the development of efficient methods for transformation and regeneration of *Tagetes*.

Another possibility would be to differentially screen the *Tagetes* cDNA library that was constructed, using probes prepared from mRNA of roots cultured in low- and normal-sulfate medium. As was shown in chapter 5, such roots differ greatly in thiophene synthesizing capacity, which is at least partially due to differences in gene expression. Although the chance of obtaining thiophene metabolism-related cDNAs with this screening procedure is still limited, it is certainly higher than with the screening procedure employed in chapter 6 since only one organ type is involved.

A classical, and possibly the best approach would be to try to purify an enzyme of thiophene synthesis which can subsequently be used to prepare

probes or antibodies for gene cloning. The enzyme(s) of ring formation would be the target of choice, since ring formation is the most characteristic reaction of thiophene synthesis. Important information with respect to the order of ring formation and demethylation was obtained in chapter 3. The monothiophene BPT, which is the natural substrate for second ring formation, can be easily labeled in, and isolated from mutant plants or root cultures. In an enzyme purification procedure this compound will be useful to monitor ring forming enzyme-activity. An assay for ring formation can be optimized in the protoplast system described in chapter 4. As was shown in chapter 2, not only roots but also young seedlings and hypocotyls can be used as a starting material for enzyme purification.

The investigations described in this thesis have provided new information about the regulation of thiophene metabolism in *Tagetes* and contribute to the development of the *Tagetes*/thiophene system as a useful model for biochemical and molecular genetic studies of secondary metabolism. The results offer several good starting points for further research, which should in the first place concentrate on enzyme purification and gene cloning.

CHAPTER 8

ALGEMENE DISCUSSIE EN CONCLUSIES

De belangstelling voor secundair metabolisme als een terrein voor biochemisch onderzoek aan planten neemt de laatste jaren sterk toe. De relatie tussen secundair metabolisme en structurele genen die coderen voor biosynthetische enzymen is eenduidig en kan onomstotelijk vastgesteld worden. Planten waarvan het secundair metabolisme vestoord is door fysiologische of moleculair-genetische manipulaties zijn meestal nog goed levensvatbaar en interessant voor nadere analyses. De effecten van manipulaties van het secundaire metabolisme kunnen direct en quantititief gemeten worden. Bovendien kunnen vele regulatoire aspecten van het secundaire metabolisme goed bestudeerd worden in cel- en weefselkweek systemen. Deze factoren maken velerlij vormen van onderzoek mogelijk naar de functie van het secundaire metabolisme. Ook kenmerken ze secundair metabolisme als een aantrekkelijk model voor de bestudering van de regulatie van metabolische processen in het algemeen en van genexpressie in het bijzonder.

De thiofenen en afrikaantjes die het onderwerp vormen van dit proefschrift, vertegenwoordigen niet alleen een interessant modelsysteem, maar verdienen ook aandacht vanwege hun specifieke eigenschappen. Afrikaantjes worden reeds sinds lange tijd gewaardeerd, zowel door bollentelers als door hobby tuiniers, vanwege de aalstjes onderdrukkende werking. Deze werking komt voor rekening van de thiofenen die voornamelijk in de wortels van afrikaantjes ophopen en die een algemene biocide werking hebben. Thiofenen vormen een van de weinige groepen van zwavel-bevattende secundaire metabolieten. De biosynthese van thiofenen omvat ten minsten twee nogal ongebruikelijke en weinig onderzochte reacties. De ene is de vorming van geconjugeerde driedubbele bindingen in lange onvertakte vetzuren. De andere is de vorming van zwavel-bevattende aromatische vijf-ringen, uitgaande van geconjugeerde driedubbele bindingen.

Bij de start van het hier beschreven onderzoek was de biosynthese route van thiofenen niet zeer gedetaileerd onderzocht. Geen van de veronderstelde

enzymen was biochemisch gekarakteriseerd. Thiofeen ophoping in planten was slecht globaal onderzocht en was niet geanalyseerd in termen van synthese, transport en afbraak. In dit proefschrift worden nieuwe resultaten gepresenteerd met betrekking tot diverse aspecten van het thiofeen metabolisme, waardoor een beter begrip is ontstaan van de regulatie van deze interessante secundaire metabolische route.

Moleculair genetische (Hoofdstuk 3) en fysiologische (Hoofdstuk 5) manipulaties werden toegepast om de thiofeensynthese-route en haar regulering te bestuderen in intacte planten en in weefselkweek. Deze onderzoekingen werden voorafgegaan door een nauwkeurige analyse van de localisatie van thiofenen op orgaan-, weefsel- en celnivo, en van de accumulatie gedurende de tijd (Hoofdstukken 2 en 4). Ook werd onderzocht in hoeverre synthese en transport bijdragen aan de uiteindelijke accumulatiepatronen (Hoofdstuk 2). In het laatste deel van het onderzoek (Hoofdstuk 6) werd een begin gemaakt met onderzoekingen naar de moleculaire regulatie van het thiofeen metabolisme.

De belangrijkste thiofenen in *Tagetes patula* en *T. erecta* zijn BBT en BBTOAc, en in beide soorten accumuleren deze stoffen voornamelijk in de wortels en het onderste deel van de stengel. In het hypocotyl zijn thiofenen vooral gelocaliseerd in de epidermis en het vaatweefsel. Een aanzienlijk deel van de thiofenen bevindt zich intracellulair, tenminste in het hypocotyl. Ook de enzymen van de thiofeensynthese bevinden zich binnen de cellen, aangezien geïsoleerde protoplasten in staat zijn tot thiofeensynthese. Daarnaast blijkt uit berekeningen dat, ten minste in het hypocotyl, een ander belangrijk deel van de thiofenen zich in de extracellulaire matrix bevindt.

Karakteristieke en verschillende accumulatiepatronen worden waargenomen voor individuele thiofenen in wortels en stengels en in de beide *Tagetes* soorten. Aangezien ze slechts moeizaam getransporteerd worden hopen thiofenen hopen op waar ze worden gesynthetiseerd. Het hogere thiofeengehalte van *T. patula* wordt verklaard door een hogere syntheseactiviteit in de

wortels en stengels van deze soort. Het lage gehalte in de bladeren is niet het gevolg van een beperkte aanvoer van sulfaat, maar van de erg lage synthese-activiteit in bladeren. De verschillen in de activiteit van het thiofeenmetabolisme tussen de twee soorten worden niet weerspiegeld in verschillen in de expressie van de genen die corresponderen met zes geselecteerde cDNA kloons. Het feit dat met alle zes cDNA kloons identieke expressiepatronen worden waargenomen in organen van beide *Tagetes* soorten duidt er eerder op dat ze op het moleculaire nivo erg overeenkomen.

In de wortels en hypocotylen van kiemende zaailingen worden snel hoge BBT concentraties bereikt. Dit verschijnsel, en de localisatie van de thiofenen in de epidermis en het vaatweefsel van de hypocotylen stemmen overeen met een functie in de afweer tegen pathogenen en predatoren tijdens de vroege ontwikkeling. De extracellulaire localisatie van een groot deel van de thiofenen vormt geen belemmering voor de toxiciteit tegen predatoren, maar kan wel een efficiente methode zijn om zelf-toxicatie te voorkomen. De extracellulaire localisatie biedt ook een verklaring voor het beperkte transport van thiofenen, aangezien dit in het algemeen via de symplast plaatsvindt.

Er werden mutanten van het thiofeenmetabolisme geïdentificeerd die C_{13} mono- en bithiofenen ophopen die niet of slechts in lage hoeveelheid voorkomen in het wild type. Voedingsexperimenten toonden bij een mutant aan dat deze gestoord is in de verwijdering van de terminale methylgroep van de monothiofeen. Deze monothiofeen, BPT, vertegenwoordigd een vertakkingspunt in de thiofeensynthese. In één route wordt de tweede thiofeenring direct in het substraat BPT gevormd, hetgeen leidt tot de vorming van MeBBT en derivaten daarvan. In de andere route wordt de terminale methylgroep eerst verwijderd, waarna vervolgens de tweede ring wordt gevormd. Deze route leidt tot BBT en derivaten daarvan. In het wild type wordt voornamelijk de tweede route gevolgd. Demethylering kan niet meer plaatsvinden wanneer de tweede thiofeenring eenmaal gevormd is.

De mutanten waren zeer belangrijk voor de verdere experimenten omdat ze het bewijs leverden dat BPT de intermediaire monothiofeen is in de

thiofeensynthese en omdat ze een ideale bron vormden van ^{35}S-gemerkt BPT, dat gebruikt werd in de voedingsexperimenten van hoofdstuk 4 en 5.

Geïsoleerde hypocotylprotoplasten zijn in staat om alle omzettingen van de thiofeensynthese te volbrengen, vanaf de vorming van de eerste thiofeenring. Geen factoren uit de extracellulaire matrix zijn hierbij vereist. De vorming van de tweede thiofeenring, hydroxylering en acetylering vinden met gelijke efficiëntie plaats in protoplasten uit de bladschede en het hypocotyl, niettegenstaande het feit dat het thiofeengehalte en de algehele *in planta* thiofeensynthese-activiteit in bladeren erg laag zijn. Uit de waarnemingen volgt dat de enzymen van de thiofeensynthese intracellulair gelocaliseerd zijn en dat een aantal van deze enzymen ook actief is in organen die weinig thiofenen bevatten.

Het thiofeenmetabolisme van transgene wortelcultures reageert veel sneller op fluctuaties in het sulfaat aanbod dan het primaire metabolisme. Zowel de algehele snelheid van de thiofeensynthese, als de snelheid van een aantal specifieke omzettingen worden verlaagd in geval van sulfaat limitatie, terwijl op het zelfde moment het primaire metabolisme niet merkbaar verstoord wordt. Voedingexperimenten suggereren dat, in geval van sulfaat limitatie, zwavelgebruikende conversies sterker omlaag gereguleerd worden dan niet-zwavelgebruikende omzettingen, en dat de vorming van de eerste thiofeenring de beperkende stap en het belangrijkste regulatiepunt van de thiofeensynthese zou kunnen vormen onder deze condities. Hertel van de thiofeensynthese activiteit na opheffing van de sulfaat limitatie treedt pas op na een overgangsperiode en dit herstel blijft volledig achterwege indien een remmer van de mRNA maturatie aanwezig is. Deze waarnemingen duiden erop dat de thiofeensynthese, ten minste gedeeltelijk, wordt gereguleerd op het nivo van de genexpressie. Verschillen in transcriptie konden niet zichtbaar worden gemaakt, nog door *in vitro* translatie, nog door Northern blot hybridisatie.

Door differentiële screening van een cDNA bibliotheek van *T. patula* werden twee cDNA kloons geïdentificeerd die corresponderen met genen welke voornamelijk in wortels en hypocotylen van *Tagetes* tot expressie komen, hetgeen tevens de organen zijn waarin de thiofeensynthese het meest actief is.

Beide kloons vertoonden geen significante homologie met bekende genen, nog op het nivo van nucleotiden, nog op het nivo van afgeleide aminozuren. De kloons zijn interessant omdat de corresponderende genen op orgaan-nivo gereguleerd zouden kunnen worden in samenhang met genen van het thiofeenmetabolisme. Binnen wortels en hypocotylen correleerden de accumulatie nivo's van de mRNA's niet met het thiofeengehalte of de thiofeensynthese-activiteit. Dit duidt erop dat binnen een orgaan de genen corresponderend met de twee kloons anders worden gereguleerd dan genen van het thiofeenmetabolisme. De genen die corresponderen met vier andere cDNA kloons komen voornamelijk tot expressie in wortels, maar niet in hypocotylen. De afgeleide aminozuur volgorden van drie van deze kloons vertonen homologie met PR-eiwitten en de vierde is een partiële ubiquitine cDNA.

Toekomstig onderzoek aan het *Tagetes*/thiofenen model zou nadrukkelijk gericht moeten zijn op de identificatie van enzymen en genen van de thiofeen-syntheseroute. Pas daarna kan de regulatie van het thiofeenmetabolisme op het biochemische en moleculair genetische nivo bestudeerd worden. Om het doel van enzym- en gen-isolatie te bereiken zijn verschillende strategieën mogelijk, waarbij de resultaten gepresenteerd in dit proefschrift waardevolle aanknopingspunten bieden.

Een mogelijkheid zou zijn de uitvoering van een mutagenese experiment, waarbij een bekend transposon of een T-DNA sequentie gebruikt worden om mutaties te genereren. Zoals werd aangetoond in hoofdstuk 3 kunnen mutanten van het thiofeenmetabolisme met een hoge frequentie verkregen worden. Een gemuteerd gen waarin een bekende DNA sequentie geïnserteerd is kan relatief gemakkelijk gecloneerd worden via diverse procedures. Mutagenese met behulp van DNA inserties vereist echter wel de ontwikkeling van efficiënte transformatie- en regeneratietechnieken voor *Tagetes*.

Differentiële hybridisatie screening van de *Tagetes* cDNA bibliotheek, waarbij probes gebruikt worden die bereid zijn van mRNA uit wortels die gekweekt zijn in laag- en normaal-sulfaat medium vormt een tweede

mogelijkheid voor directe genklonering. In hoofdstuk 5 is aangetoond dat zulke wortels sterk verschillen met betrekking tot thiofeensynthese-activiteit, hetgeen, tenminste gedeeltelijk, berust op verschillen in genexpressie. Omdat de beide probes die worden toegepast afkomstig zouden zijn van hetzelfde orgaan-type (wortels), biedt deze werkwijze een aanmerkelijk grotere kans op de identificatie van cDNA's die daadwerkelijk gerelateerd zijn aan thiofeensynthese dan de aanpak zoals beschreven in hoofdstuk 6.

Een klassieke, en mogelijk de beste aanpak zou zijn om te proberen een enzym dat betrokken is bij de thiofeensynthese op te zuivering met behulp van biochemische technieken. Het gezuiverde enzym zou vervolgens gebruikt kunnen worden om probes of anti-lichamen te produceren, met behulp waarvan het corresponderende gen gekloneerd kan worden. Een (het) ringvormend(e) enzym zou als eerste voor opzuivering in aanmerking komen omdat ringvorming de meest karakteristieke reactie uit de thiofeensynthese is. Belangrijke informatie met betrekking tot de volgorde van ringvorming en demethylering werd verkregen in hoofdstuk 3. De monothiofeen BPT, het natuurlijke substraat voor de vorming van de tweede thiofeenring, kan gemakkelijk gelabeld en geïsoleerd worden uit mutant weefsel. In een enzymzuiveringsprocedure kan deze stof gebruikt worden om de enzymatische activiteit te volgen. De kondities voor een assay om ringvorming te meten kunnen geoptimalizeerd worden in het protoplastensysteem, zoals beschreven in hoofdstuk 4. Niet alleen wortels, maar ook jonge zaailingen en hypocotylen zouden gebruikt kunnen worden als uitgangsmateriaal voor enzymzuivering.

Het hier beschreven onderzoek heeft nieuwe inzichten opgeleverd met betrekking tot de regulatie van thiofeensynthese in *Tagetes* en draagt bij aan de verdere ontwikkeling van het *Tagetes*/thiofeen systeem als een bruikbaar model voor biochemisch en moleculair genetisch onderzoek van het secundaire metabolisme. De resultaten bieden goede aanknopingspunten voor verder onderzoek, waarbij enzymzuivering en genklonering in eerste instantie centraal moeten staan.

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CURRICULUM VITAE

Jonny Johannes Marie René (John) Jacobs werd geboren te Nuth op 28 december 1961. Hij volgde de Atheneum (B) opleiding aan het St. Janscollege te Hoensbroek van 1974 tot 1980. In dat jaar begon hij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Tijdens de afstudeerfase heeft hij twee hoofdvakken gedaan bij de vakgroepen Erflijkheidsleer, onder begeleiding van professor Maarten Koornneef, en Moleculaire Biologie, onder begeleiding van Dr. Pim Zabel. Ook werd een bijvak gevolgd bij de vakgroep Plantenfysiologisch onderzoek, onder begeleiding van professor Vredenberg. Tenslotte verbleef hij gedurende zes maanden in de Verenigde Staten voor een fytopathologische stage aan de Michigan State University, onder begeleiding van Dr. Shauna Somerville. De studie werd in maart 1987 afgesloten, waarna gedurende 19 maanden de vervangende dienstplicht werd vervuld bij het CPRO te Wageningen (toen SVP; Stichting voor Plantenveredeling). In deze periode werd onderzoek gedaan naar moleculair genetische merkers in aardappel, in de groep van Dr. Henk Huizing en Dr. Frans Krens. In juni 1989 trad hij in dienst van de Katholieke Universiteit Nijmegen als Assistent in Opleiding (AIO) bij de vakgroep Experimentele Plantkunde onder leiding van professor George Wullems. In de secundaire metabolietengroep van Dr. Ton Croes verrichtte hij onderzoek naar de regulatie van het thiofeen-metabolisme in twee *Tagetes* soorten (afrikaantjes). Tijdens de aanstelling heeft hij tweemaal geassisteerd bij het practicum Biotechnologie II. Ook volgde hij gedurende drie weken de cursus "Molecular and Developmental Biology of Plants" in het Cold Spring Harbor Laboratory te New York. Per 1 december 1994 heeft hij een aanstelling als Post-Doc aan het INRA te Versailles.

LIST OF PUBLICATIONS

Jacobs JJMR, Krens FA, Stiekema WJ, van Spanje M, Wagenvoort M (1990) RFLP analysis in *Solanum* spp for the construction of a genetic map of *Solanum tuberosum* L : a preliminary study. Potato Research 33: 171-180.

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